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Neal T. Williams (MS, Chemical Engineering)

Continuous Production of RNA using a Stir-cell Bioreactor

Thesis directed by Professor Robert H. Davis

The increased study of ribonucleic acids (RNAs) due to the discovery of their potential catalytic and selective binding properties has prompted research into developing a large-scale, cost-effective RNA production strategy. Unfortunately large amounts of RNA, made either by direct chemical or enzymatic synthesis, are expensive and difficult to produce because of the high material cost and limitations found in batch mode processes. In an effort to make large amounts of RNA efficiently and effectively, a process using immobilized DNA templates and bacteriophage T7 polymerase in a continuous stir-cell membrane bioreactor has been investigated. This method decreases the overall cost of the process by retaining the polymerase and template while separating the newly formed RNA using an ultrafiltration membrane. This membrane has a molecular weight cut-off of 100,000 daltons and is a low protein binding, hydrophilic, regenerated cellulosic disk. Membrane studies indicated that the transmission of RNA is affected by the transcription mixture.

Further studies of the stir-cell bioreactor indicated that the polymerase is possibly denatured causing the transcription reaction to plateau. The continuous addition of precursor nucleotide triphosphates and buffer does not prevent the reaction from stopping, but the addition of polymerase causes in increase in RNA production. Optimized NTP concentrations produce more RNA with less abortive transcripts compared to the products of reactions with limiting NTPs. Continuous production of RNA is a viable alternative for its large scale production. Reactions have been performed with a continuous feed stream of NTPs and buffer for up to 300 minutes. A continuous 1.0 mL transcription reactor with a feed of 0.85 mL/hr produced 60 nanomoles of RNA in 300 minutes using 20 μL of .023 mg/mL stock solution of T7 polymerase. This is about 230 mg of RNA.

DEDICATION

This work is dedicated to my wife, Sheryl, for her understanding, encouragement, love, and support.

CONTINUOUS PRODUCTION OF RNA USING A STIR-CELL MEMBRANE BIOREACTOR

by

Neal T. Williams

B.S., United States Air Force Academy, 1993

A thesis submitted to the

Faculty of the Graduate School of the

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of the requirements for the degree of

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Department of

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CHAPTER I

INTRODUCTION

1.1 Nucleic Acids and RNA Transcription

There are two groups of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Nucleic acids are linear polymers of nucleotides. Nucleotides are composed of a phosphate group attached to the 5' carbon of a sugar with a purine or pyrimidine base attached to the 1' carbon (Figure 1.1). The bases include adenine (A), thymine (T), guanine (G), cytosine (C), or uracil (U), with uracil being unique to RNA and thymine being unique to DNA. The nucleotides are linked by a phosphodiester bridge through the 3' and 5' position of successive sugar residues (1). DNA and RNA differ in two structurally significant ways: in DNA, a 2' hydroxyl group on the RNA is missing, and uracil is substituted for thymine in RNA. Double-

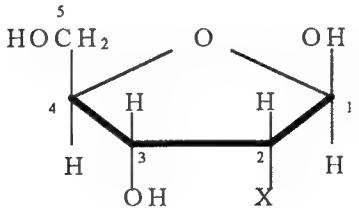


Figure 1.1 The ribose sugar unit found in nucleic acids. In DNA the X is hydrogen while in RNA, X is a hydroxyl group. The numbers indicate the carbons in the sugar and the backbone of nucleic acids is linked between the 5' and 3' carbons.

stranded nucleic acids form a double helical structure, with the sugar and phosphate serving as the backbone and the purine and pyrimidine bases forming an inside ladder-like link with adenine and thymine forming two hydrogen bonds while cytosine and guanine form three hydrogen bonds (Figure 1.2).

In nature, DNA is the template from which RNA molecule sequences are determined. DNA is first unwound from its double helical structure, which allows the internal bases to be exposed, then an enzyme replicates a new RNA molecule. The process of making RNA from DNA is called transcription. The new RNA molecules serve a varied role within the cell. There are the messenger RNAs, which contain information for the

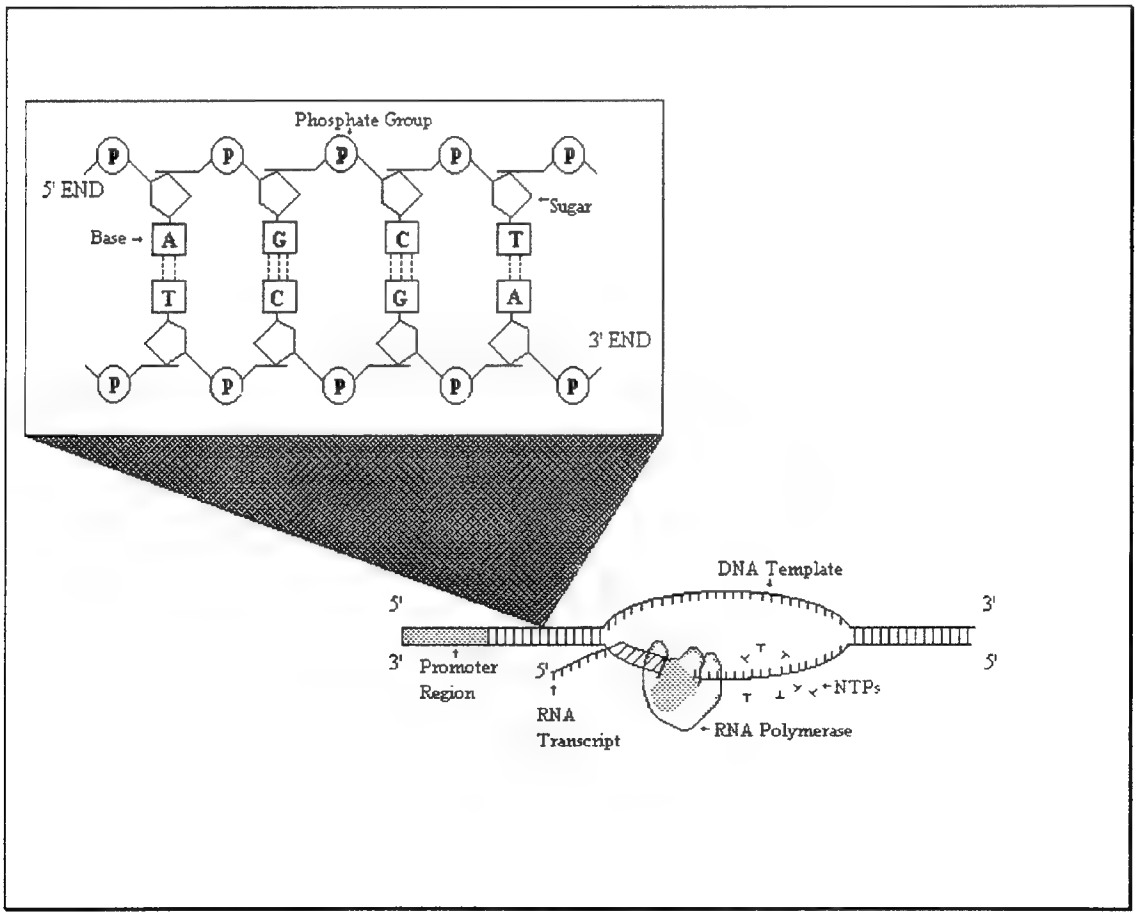


Figure 1.2 RNA Transcription and a Visual of DNA (2)

amino acid sequence, the ribosomal RNAs, which help to translate the messenger RNA, and finally the transfer RNAs, which recognize certain amino acids and bring these protein pre-cursors to the messenger RNA codon sites. The process called translation, which is responsible for the development of proteins, occurs when the messenger RNA is read by the ribosomal RNA (3). This process from which DNA is transcribed to form RNA which is then used for the translation of protein is known as the central dogma of molecular biology (4).

The importance of nucleic acids is two-fold. First, DNA is the hereditary molecule of all life. The second importance involves recent discoveries concerning RNA, demonstrating that single-stranded RNAs have potential pharmaceutical value because of their catalytic and/or selective binding properties. It has been demonstrated that RNA can catalyze reactions *in vivo* much like protein enzymes, which until a decade ago were the only known biocatalytic agents. These specific RNA molecules can catalyze cleavage as well as joining reactions (5). Due to its ability to cleave and join, catalytic RNA "ribozymes" could have future *in vivo* applications, such as the degradation of specific RNAs from the DNA genome (6). These RNA molecules, known as ribozymes, can cleave their RNA substrates using transesterification or phosphoester transfer reactions. Furthermore, a process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) has demonstrated that high affinity ligands, such as RNA, can bind to reduce the activity of target proteins such as HIV reverse transcriptase (7,8). These binding properties have increased interest in using RNA for *in vitro* research, diagnostics and therapeutic applications (9,10). In addition, the inhibition of certain genes has been demonstrated by expression of antisense RNA in mammalian cells, plant protoplasts, and transgenic plants (9). These discoveries have created a need for RNA for which further studies can be continued.

There is a need for an efficient and effective process for producing milligram or greater amounts of RNA. Currently, there exist two ways of producing RNA. The

first method is the production of RNA by direct chemical synthesis; this is ideal for short RNA molecules, but the process becomes prohibitively expensive as the size of the molecule increases due to exponentially decreasing yields (11). Direct chemical synthesis of RNA is similar to the production of synthetic DNA, which is accomplished base by base. Unfortunately, RNA also contains a 2' hydroxyl group which needs to be protected so that branching does not occur. This added step in chemical synthesis, of protecting the hydroxyl group, increases the cost to make a linear chain of RNA.

An alternative method of producing RNA is to copy the way nature makes RNA. This is done with a protein enzyme called RNA polymerase. The polymerase is able to read DNA and then using appropriate reactants, is able to make a complementary molecule of RNA. This process, called transcription, produces RNA in batch reactors using synthetic DNA templates and an RNA polymerase (11,12). Unfortunately, this method is costly due to the expense of the initial materials, DNA template, and polymerase. Low yields of the specific product are seen using this production method, because of the large number of aborts (short RNA transcripts) that the polymerase produces, but this process can produce an RNA molecule of any size. The transcription reaction is a process that occurs *in vivo* or *in vitro*. A DNA template that contains a specific double-stranded promoter base sequence which a DNA-dependent RNA polymerase can recognize and bind to, is the molecular machinery behind this method (Figure 1.2). The polymerase initiates transcription beyond the promoter and begins to synthesize an RNA molecule complimentary to the DNA template. The polymerase uses nucleoside triphosphate (NTP) monomers to build the desired RNA molecule. When the transcript is finished, the polymerase and RNA molecule dissociate from the DNA template.

The polymerase of interest in this research is the bacteriophage T7 RNA polymerase. The T7 RNA polymerase is an enzyme that carries out promoter-specific,

DNA-directed RNA polymerization *in vivo* and *in vitro* as a 98,856 dalton monomer (13). The crystal structure reveals a molecule with a deep cleft that can recognize a double-stranded DNA template (14). Once the T7 recognizes the promoter sequence, it begins an initial abortive phase of transcription in which short (2-9 bases) transcripts are made and released (15). It then proceeds to elongate, which involves a conformation change in the enzyme and its release from the promoter sequence. The polymerase proceeds down the DNA template, obtaining the required complementary NTPs from the surrounding solution. Upon completion, the polymerase detaches and the newly completed RNA transcript separates. A by-product of this process is pyrophosphate, which hydrolyzed released from the nucleoside triphosphates. Once incorporated, the nucleotide becomes a monophosphate. It has been seen that this by-product is a possible inhibitor of transcription (16).

1.2 Bioreactor Design

A method has been described to make RNA of a defined length and sequence using T7 RNA polymerase and templates of synthetic DNA which contain the T7 promoter (17). To successfully scale this process into making milligram and larger quantities, the process must take into account the fact that DNA template and polymerase are costly. A goal of transcription reactor design, therefore, is to reuse these two species.

Using this idea as a basis, a scale-up design has been investigated by attaching the DNA templates to biotin which binds to streptavidin-coated agarose beads (18). The avidin-biotin interaction is the strongest known noncovalent reaction between protein and ligand, with a K_a of $1x10^{-15}$ M (19). This immobilization of the DNA template on the agarose beads allows isolation of the two components, the template and newly formed RNA transcripts. Therefore, the templates can be reused over successive rounds of transcription.

Without immobilization, the template is simply used and lost after transcription, because there is no easy, inexpensive way to recover the solution-phase DNA template. Retention and re-use of the DNA template makes the process become more cost effective. Furthermore, this process has been shown to successfully produce RNA within a stir-cell reactor (Figure 1.3) using an ultrafiltration membrane to separate the completed RNA transcripts from the agarose beads containing the DNA templates and the polymerase (20). Initial research on producing RNA in a semi-continuous, immobilized reactor focused on determing the amount of RNA that could be produced in the stir-cell compared to batch reactions, comparing solution-phase template RNA production with that from immobilized templates, and finding a membrane which allows for the transmission of RNA and the retention of the polymerase and beads. This research showed that the stir-cell is a viable alternative to batch reactions. The immobilized template functions in the stir-cell and is more productive than the solution-phase templates which are lost through the membrane over time. Finally, the results showed that an ultrafiltration membrane with a molecular weight cut-off of 100,000 is sufficient for the retention of the polymerase and the transmission of newly formed RNA molecules.

The stir-cell reactor shown in Figure 1.3 is a 3 mL vessel that has a magnetic stir-bar impeller on a vertical shaft that rotates when the reactor is placed on a magnetic stir plate. The membrane is located below this impeller, allowing for a crossflow or tangential flow across the membrane to prevent concentration polarization. The manufactured purpose of this device is for concentration of proteins and diafiltration. Although designed to be operated either semi-continuously or continuously, the process of producing RNA needs a modified design due to limitations imposed by the original design.

Another goal of scale-up is to increase yield (amount of product produced per amount of raw material utilized) rather than productivity (amount of product per

volume per time), because in order to optimize the production of RNA the cost of producing RNA must be maximized. The primary cost is the starting materials which include the DNA template, polymerase and NTPs. An objective of optimizing the

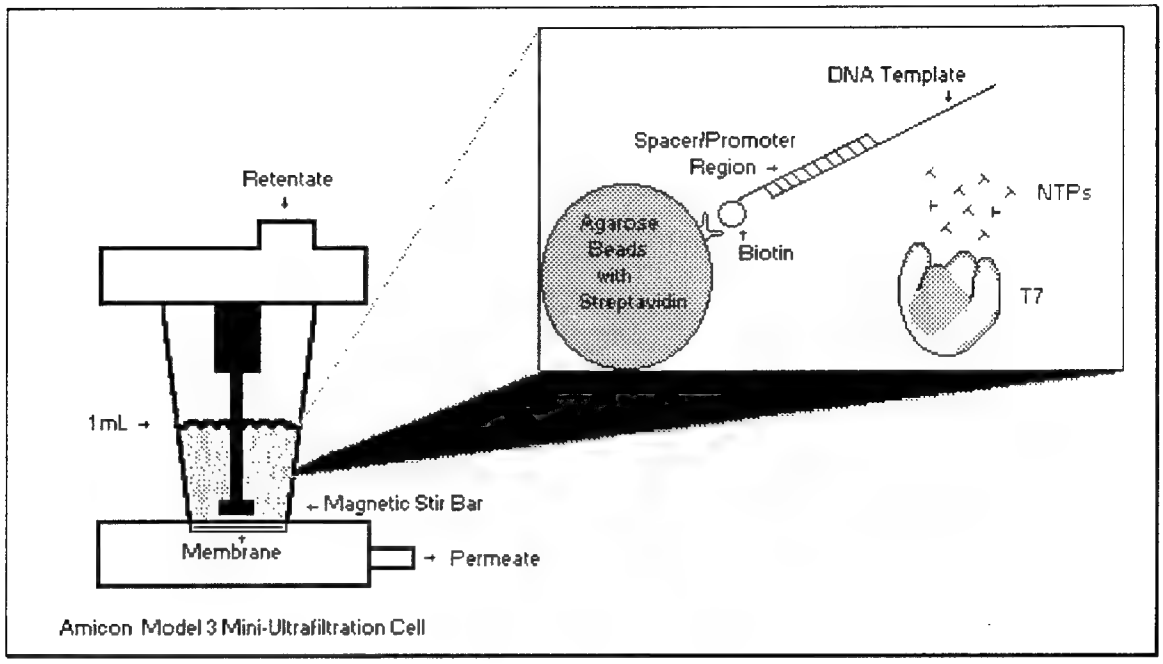


Figure 1.3 Stir Cell Design and a Visualization of the Transcription Process

yield is to maximize the amount of specific RNA transcripts, as opposed to the more prevalent aborts, to reduce downstream processing required to obtain the desired product.

1.3 Ultrafiltration

Membranes represent an emerging technology, and, because of their wide range of properties, a large number of separation processes are available. Membranes are permselective barriers between two phases. Phase one is the upstream or retentate side, while phase two is the downstream or permeate side. In this research, the retentate side ideally retains the DNA template, polymerase, and reactants, while the

newly formed RNA transcripts are transmitted to the permeate side. Separation can occur because the membrane allows the transport of one component from the retentate more readily than other components in the mix. Differences in the size, pressure, affinity, charge, or chemical properties of the molecules dictate the ability of the membrane to separate components (21). The benefits of membrane technology include continuous separation, low energy consumption, ability to be combined with other separation processes, separation under mild conditions, easy up-scaling, adjustable variable membrane properties, and the fact that no additives are required. The drawbacks include concentration polarization, membrane fouling, low membrane lifetime, high membrane cost, and low selectivity (22).

An integral part of the bioreactor production scheme is the use of an ultrafilitration membrane to separate the RNA transcripts from the original materials. The process depends on the ability of a permeable membrane to differentiate between solutes of different sizes (22). The membrane is like a sieve with extremely small pores. The selection of a membrane with a molecular weight cut-off (MWCO) that is able to pass the RNA transcripts while retaining the DNA and polymerase is essential for a cost-effective bioreactor. MWCO is defined as the approximate molecular weight of a globular solute which is 90% rejected by the membrane, with rejection also affected by the actual molecular shape (22). Nucleic acids, which are characterized by their length in bases or base pairs, have little or no tertiary structure like proteins but they do have secondary structure. The structure of proteins is defined first by its primary structure, which is the amino acid sequence. The secondary structure is the interaction of the amino acids with one another by hydrogen bonding in a single domain characterized by alpha helices and beta-pleated sheets. Nucleic-acid secondary structure is similar to that of proteins, but the structure is formed by the hydrogen bonding of single-stranded linear regions of RNA or DNA forming complementary Watson-Crick base-pairing with bases on an opposing strand. Finally, tertiary

structure is how the entire protein packs itself, forming interactions between many amino acids that are not in linear sequence with respect to each other (1). Studies have shown that the MWCO of the membranes compared to the length of single-stranded and double-stranded DNA is comparable to that of proteins of the same size (23). Membrane technology is important in this scheme is because the process of separation is achievable at modest temperature, low pressure, sterile conditions, and in batch or continuous mode (24). These conditions are vital for nucleic acids which are extremely sensitive to temperature and in need of a clean environment without the possibility of hydrolytic enzymes.

Ultrafiltration membranes are developed from polymeric materials such as polysulfone, polyether sulfone, polyacrylonitrile, and cellulosic materials, as well as from inorganic materials such as alumina (21). These synthetic membranes can be grouped by various characteristics: 1) non-sorptive (e.g., regenerated cellulose) or sorptive (e.g., polysulfone) and 2) symmetric and asymmetric. Ultrafilitration membranes have the typical structure of asymmetric membranes. This structure is characterized by a thin top layer supported by a more porous sublayer (25). The top layer defines the resistance to mass transfer, whereas the sublayer provides mechanical strength. The thickness, pore size distribution, and surface porosity of the top layer characterize the membrane separation properties. Pore diameters of ultrafiltration membranes typically range from 20 to 1000 Angstroms (21).

During separation, the ultrafilitration membrane's performance decreases with time. This is seen by a decrease in flux and possible selectivity. Reasons for this decrease are concentration polarization and fouling. Concentration polarization occurs when solutes or particles that are retained by the membrane gather at the surface or in the boundary layer next to the membrane surface (26). This accumulation of particles at the surface acts like a secondary membrane. This is referred to as a gel layer, and the formation of this layer can significantly decrease the flux. The secondary

membrane might also have a higher retention than the original membrane, which can lead to a higher retention of the material which is to be separated from the mixture. Fortunately, concentration polarization can be reduced by using a dilute solution, stirring, or adding a tangential flow of solution across the membrane surface (27).

Another cause of decreased flux is fouling of the membrane. Fouling occurs in one of two ways. The first is that the pores become blocked at the membrane surface by particles which accumulate at the surface and are larger than the pores. The second is that particles can accumulate within the pores and cause the interior of the pores to shrink. This can occur by adsorption, precipitation, and chemical reaction. One way to reverse the affects of fouling is to vigorously clean the membrane after each separation. Modification of the membrane surface chemistry or of the pH or ionic strength of the solution can also affect membrane fouling.

1.4 Introduction to Thesis Research

It has been shown that RNA transcription is possible with the use of a stir-cell bioreactor and an ultrafilitration membrane to separate the initial components from the newly formed RNA transcripts (20). This research needs to be continued to further validate the use of a bioreactor for the production of RNA. The previous research in this area only concluded that producing RNA in a reactor was possible. Areas that needed to be further clarified were why the reaction decreases in the bioreactor over time and why the stir-cell is not as efficient as a batch reactor. Finally, a more in-depth study of RNA transcription in the stir-cell needed to be performed to better characterize RNA transcription within the stir-cell, with the hope of optimizing the reaction and going to a continuous-flow mode of operation. The purpose for such research is to continue investigating the possible use of a membrane bioreactor for producing RNA in large amounts using immobilized DNA template and T7 RNA polymerase. Characterizing the transcription reaction in the reactor is important for

understanding how to potentially optimize the reaction so that large amounts of RNA can be made cost effectively. Furthermore, studying the reaction in a semi-continuous mode before scaling up is important in determining whether this method has a potential to outperform solution-phase RNA transcription, which is the current method of choice in making large amounts of RNA. From this characterization, the ultimate goal is to design a bioreactor in which reaction materials can be continuously fed and RNA continuously removed while re-using the more expensive starting materials (DNA template and polymerase). The value of such a system is the potential to make large amounts of RNA efficiently and effectively. Since it has been shown that RNA is a potential pharmaceutical the need exists for a method to produce RNA.

Chapter 2 describes the use of radioactive tracer RNA molecules to better understand fouling of the membranes in a semi-continuous mode. Characterizing this fouling is important in understanding the productivity of separating RNA from the initial materials. The results of these experiments indicate potential causes of fouling and track the retention and transmission of the RNA molecules.

The fouling which was found needs to be characterized and understood before scale-up can continue. To understand this problem, an in-depth study of the reaction materials and how they react in the stir-cell bioreactor is described in Chapter 3, with the underlying goal of characterizing the effects of the agarose beads, the polymerase, and RNA within the reactor.

In order to understand how the transcription reaction affects the membrane and how RNA is transmitted through the membrane, Chapter 4 characterizes the production of RNA within the stir cell, with the results indicating how best to approach the problem of optimizing the transcription reaction as well as studying the potential problems that could occur when scale-up to continuous flow mode begins. Through these results, a new modified design for a bioreactor was made to overcome the limitations inherent to the typical stir-cell reactor.

Next, the experiments described in Chapter 5 utilized the newly designed bioreactor and investigated how the reaction occurs in a continuous-flow mode. With this continuous process, the production of RNA needs to be clearly understood and the determination made of whether flux decline is a potential problem. The final chapter gives conclusions and recommendations for the future study of the continuous-flow mode of RNA transcription production to make large amounts of RNA.

CHAPTER II

CHARACTERIZATION OF NUCLEIC ACID ULTRAFILTRATION

2.1 Objective

The objective of the ultrafiltration studies was to characterize the transmission of RNA molecules of various sizes and shapes in a buffer solution through ultrafiltration membranes. For comparison, studies were also done to determine the effects of transmission when other components of the transcription mixture were present. A stir cell with an accompanying ultrafilitration membrane was used. The ultrafiltration studies used two types of membranes to study the transmission of RNA. The first type was a YM membrane which is an advance hydrophilic membrane with low non-specific protein binding properties. This membrane, in the 100,000 molecular weight cut-off (MWCO) range, has been determined previously to be the optimal membrane (20). The 30,000 MWCO of this type of membrane was also studied. This YM100 membrane (Amicon, Beverly, MA) was used predominantly in the following experiments with additional studies using an XM membrane with MWCO of 50,000 and 300,000. The XM membranes differ from the YM membranes in that they are only moderately hydrophilic and non-ionic. These membranes were used in the studies described in Chapters 3 and 4 for separating newly formed RNA transcripts from the transcription reaction. Furthermore, the studies indicate the feasibility and possible shortcomings inherent in using such a process.

2.2 Materials and Methods

2.2.1 Formation of DNA Template

2.2.1.1 Immobilized DNA Template for UU-Hairpin

A template coding for a UU hairpin was obtained from Macromolecular Resources (Fort Collins, CO) and consisted of two complementary DNA strands, a 36-nucleotide top strand and a 48-nucleotide bottom strand. These synthetic oligodeoxynucleotides were prepared by the company using conventional phosphoramidite chemistry. The template contains a double-stranded region which is designed with a Class III T7 consensus promoter sequence downstream. The bottom strand codes for a hairpin shaped, 3783.4-dalton, 12-nucleotide RNA molecule, as shown in Figure 2.1. This dodecamer sequence forms a hairpin structure containing a CUUG loop motif at low salt concentrations and a base-paired dimer with UU bulges at high salt concentrations.

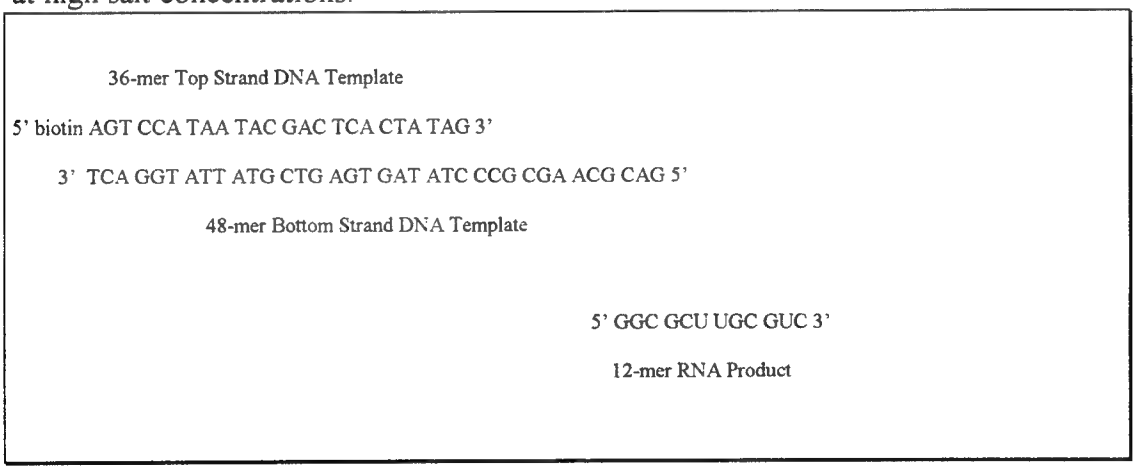


Figure 2.1 Sequence of UU Hairpin DNA template and corresponding RNA product

The top strand contains a terminal biotin group on the 5' end. The biotinylated DNA top strand template was immobilized on streptavidin-coated agarose beads by

adding 7500 picomoles of DNA top strand to 1.0 mL of 50 % v/v streptavidin-agarose beads (Pierce Immunochemicals; Rockford, IL) (18). This mixture was incubated for 16 hours at 4° C on a Labquake® Shaker rotary mixer (Labindustries, Inc.; Berkeley, CA) After incubation, the beads were spun down using a centrifuge, and the supernatant was carefully withdrawn from the beads, making sure no beads were removed. The beads were then washed four times (400 μL) with sterile, deionized water (d_AH_2O), and then the bottom strand was added to the beads and allowed to hybridize for 24 hours at 4° C. The bottom strand was added with a 1.5X molar excess to assure that the bound top strand underwent hybridization. The supernatant and washes were quantified spectrophotometrically (Hewlett Packard 8452A Diode Array Spectrophotometer; Palo Alto, CA) at 260 nm to determine the amount of bound top strand DNA, which typically was 5,000 to 7,000 picomoles of DNA retained to the beads. This corresponds to a concentration of about 10 to 14 μM. After hybridization, the beads were washed and resuspended in d_AH_2O for a final volume of 1.0 mL. The beads were stored at 4° C on a rotary mixer that keeps the beads from sedimenting in the storage vessel. The device rotates with the sample clipped to the rotating bar and the continuous rotation keeps the beads mixed in the eppendorf tube so that the beads remain in solution; otherwise the beads will sediment.

2.2.1.2 Solution Phase Pseudo-knot and Rigid Rod DNA Template

The DNA templates for the pseudo-knot and rigid-rod RNAs were obtained from Macromolecular Resources (Fort Collins, CO) and consisted of two complementary DNA strands for each specific template. These synthetic oligodeoxynucleotides were prepared by the company using conventional phosphoramidite chemistry. The templates were based on the design described in Section 2.2.12. The pseudo-knot template codes for a 8644.6 dalton, 28-nucleotide

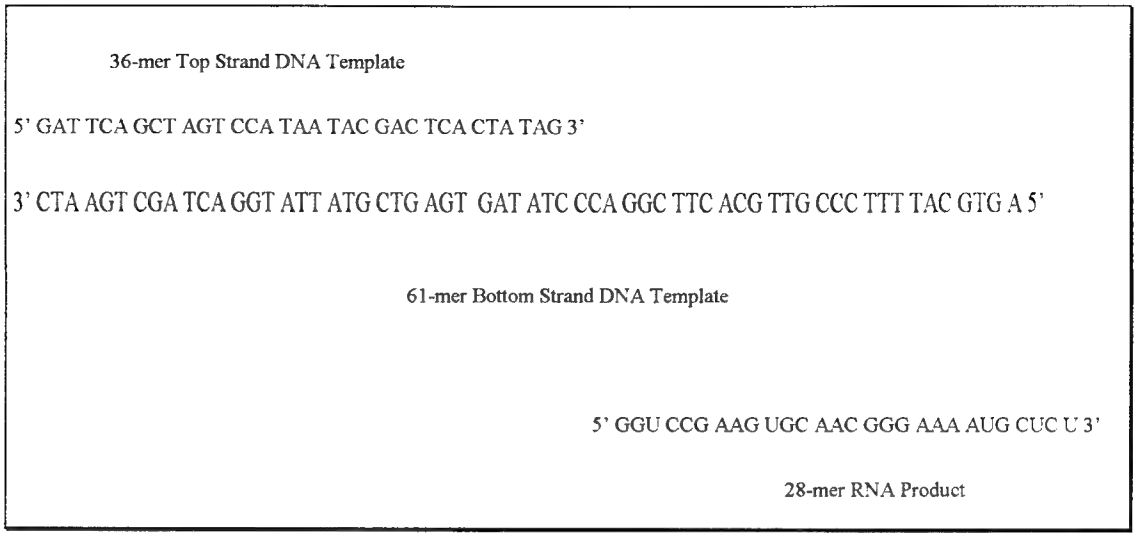


Figure 2.2 Sequence of Pseudo-knot DNA template and corresponding RNA product

psuedo-knot RNA. This RNA molecule was discovered through SELEX and binds to the human immunodeficiency virus reverse transcriptase (HIV-RT) primer binding site (8). The templates used for the formation of the 28-mer can be seen in Figure 2.2.

The template for the rigid-rod RNA codes for a 9578.2 dalton, 31-nucleotide RNA which was designed to be a linear form of single-stranded RNA in solution without forming any secondary structure. The template and product can be seen in Figure 2.3. Hybridization of the DNA templates was done without immobilization to

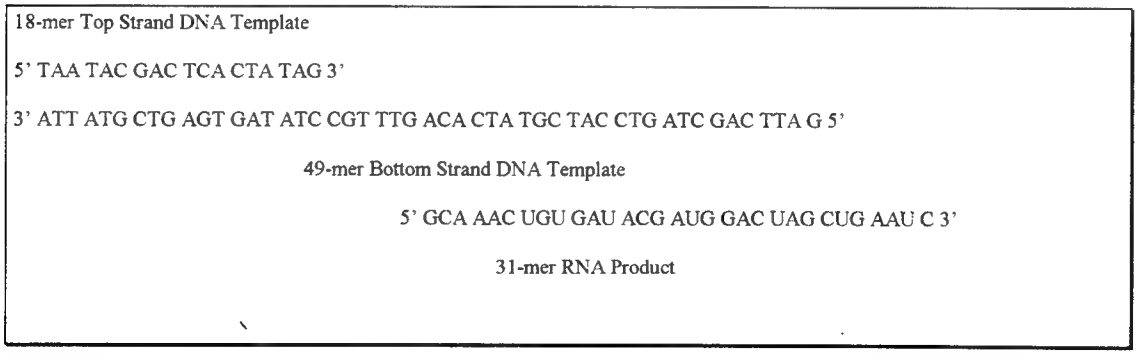


Figure 2.3 Sequence of Rigid Rod DNA template and corresponding RNA product

the streptavidin coated agarose beads. Solutions of the top template and the bottom template that codes for the 31-mer product, both at 10 μM, were mixed at 90° C for 5 minutes and then immediately put in ice for 30 minutes. This procedure was used to hybridize the two templates for use in a solution-phase T7 RNA polymerase transcription reaction.

2.2.2 Labeled RNA Preparation, Purification and Recovery

2.2.2.1 Labeled UU Hairpin RNA Preparation

A 250 μL batch transcription reaction was performed, incorporating radioactively labeled uridine triphosphate ([α-^{32}P]-UTP, 800 Curies/millimole, DuPont/NEN; Wilmington, DE) into RNA products, to obtain a labeled RNA UU Hairpin (12-mer) molecule. An immobilized hairpin template, as described in Section 2.2.1.1, was used in this transcription reaction. A 250 μL transcription was set up in a 2.0 mL reaction vessel. The reaction mixture contained 1 μM DNA template, 40 mM Tris-HCl (pH 8.1), 20 mM Magnesium Chloride, 5 mM Dithiothreitol, 1 mM Spermidine-HCl, 0.01% Triton X-100, 1.0 mM ATP, GTP, and CTP each, 0.1 mM UTP, 0.125 μM [α-^{32}P]-UTP (10 μCi), and 0.023 mg/mL T7 polymerase. This reaction mixture used d_AH_2O to bring the volume up to the required level.

The reaction was incubated for 4 hours at 37 degrees Celsius and continuously mixed on a rotary mixer (Labindustries, Inc.; Berkeley, CA). After 4 hours, the beads were sedimented in a mini-centrifuge for 1 minute (Tomy® HF-120 Capsule Mini-centrifuge™; Palo Alto, CA) and the supernatant was removed. The supernatant was then resuspended with a 1/10 supernatant volume of sodium acetate and 6 X volume of ethanol overnight at 4° C. These supernatant samples were centrifuged for 50 minutes at 14,000 rpm. The resulting pellet was resuspended in d_AH_2O and 75%

formamide and then loaded on an analytical polyacrylamide gel for characterization by electrophoresis.

A 40% acrylamide stock solution was made up by dissolving 380 g of acrylamide and 20 g of bis-acrylamide in 1 liter of deionized water. The acrylamide solution used for the formation of the gel was then formed by making a 20% solution from the stock acrylamide solution and then adding 8 M Urea and 10 X of TBE gel. This acrylamide was added to a beaker (50 mL), and then 250 μL of 10% ammonium peroxydisulfate (APS) and 100 μL of TEMED were added. These last two materials induce polymerization. This solution was then quickly poured between two clean glass plates separated by a 1.5 mm spacer, sealed with a rubber gasket, and held together with metal clamps. A 4-lane comb was inserted into the top immediately after pouring.

Once the gel was polymerized the clamps, comb and gasket were removed and the assembly was placed in a gel electrophoresis box. A 1X TBE solution was then inserted into the top and bottom electrode wells of the gel box, making sure no bubbles formed between the interface of the gel and buffer. A stock solution of electrophoresis buffer (TBE) was prepared by dissolving 890 mmoles of Tris Base, 890 mmoles Boric Acid, and 20 mM of Ethylenediaminetetraacetic acid (EDTA) in 2 liters of water. This solution was then autoclaved. The gel was pre-electrophoresed for 10 minutes, and then each well was cleaned using a syringe. The samples were then added to the middle lanes, and a bromophenol blue solution and a xylene blue dye solution were added into the outside lanes. The gel was connected to the power supply (BioRad PowerPac 3000; Richmond, CA) for up to 4 hours at 550-650 volts or until the bromophenol blue solution had moved to within about 3 inches from the bottom of the gel assembly.

After the dye traveled the required distance, the power supply was switched off, the buffer was removed, and the gel assembly was carefully removed from the box.

The spacers were first removed, and then the glass plates were removed very carefully. The top plate was removed first, then a piece of plastic wrap was placed on the gel. Once the plastic wrap was on the gel, the bottom plate could be removed.

The gel-fractionated RNA products were then visualized with X-ray paper. The gel was put in an exposure cassette along with an undeveloped piece of X-ray paper. The gel and X-ray were marked paper so that they could be put back together again for visualizing where the desired RNA product was located. The X-ray paper was exposed to the gel for 5 minutes and then removed. It was then put in a solution of Developer until the image appeared and then finally into the fixer. Using the developed X-ray picture, the print was placed back onto the gel. Then using a backlight, the correct band on the gel was outlined and cut out to obtain the desired RNA product (12-mer).

The cut-out gel band was broken up into minute pieces and placed in a 10 mL RNA elution buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. After overnight agitation, the acrylamide particles were filtered out using glass wool and the sample was concentrated in a vacuum centrifuge (Speed Vac® Plus SC110A, Savant Instruments, Inc., Farmingdale, NY). The resulting pellet, which was formed by drying (dewatering) the sample in the vacuum (dehydration), was resuspended in d_AH_2O and then purified using Sephadex G-25 beads in a 7 mL column. The column was made with a 10 mL pipette, with glass wool stuffed into the base of the pipette. The beads were then added continuously to form a continuous layer of beads with no bubbles. Once the beads were loaded, 10 volumes of d_AH_2O were passed through to equilibrate the column. Using a 0.01 M $(NH_4)_2CO_3$ (pH 8.0) buffer, the resuspended RNA was loaded, eluted with the buffer, and collected in 200 μL fractions. Once the RNA was put on the column, the buffer had to be continuously fed into the column so that the RNA was continuously moving through the column. The resulting fractions were then held up to a Geiger counter and the radioactive fractions were pooled

together and concentrated in the vacuum centrifuge. The resulting pellet was then resuspended in 400 μL of d_AH_2O. The reason for this step is to de-salt the purified labeled 12-mer RNA and to remove any other contaminants still located with the RNA.

2.2.2.2 Synthesis of Labeled Pseudo-knot and Rigid Rod RNA

The templates described in section 2.2.1.2 were placed in two separate 250 μL transcription reactions containing the components described in section 2.2.2.1. The two RNA molecules formed were the 28-mer pseudo-knot and the 31-mer rigid rod transcripts.

2.2.3 Semi-Continuous Stir Cell Experiments

2.2.3.1 Buffer Experiments

A YM100 membrane (Amicon: Beverly, MA) was prepared by rinsing the membrane, which was accomplished by floating the membrane skin (glossy) side down in a beaker of distilled water for at least one hour, changing the water three times. This same procedure was used for the YM30, XM50, and XM300 buffer experiments. The membrane was then placed glossy side up within the stir-cell bioreactor (Amicon; Beverly, MA) which was filled with 1.2 mL of buffer (10 mM Tris-HCl (pH 7.5) and 1.0 mM EDTA). The YM100 is a hydrophilic membrane consisting of a very thin film (100-400 Å) which has the controlled pore structure. This thin film is supported by a thicker (50-250 μm) open spongy layer (Amicon). The membrane is made of regenerated cellulose with the designation YM, a company designation, and the 100 is the MWCO of the membrane divided by 1000. Next, 10 μL of radioactively labeled RNA (12-mer) was added to the reactor at a concentration of 3.34 μM. A 100 μL

retentate sample was taken at the zero time point. The mixture was allowed to sit with the stir bar rotating at an approximate rate of 150 rpm. Every hour for six hours, 500 μL of permeate was withdrawn through the membrane using a syringe and 100 μL of retentate was taken from the stir-cell. While the samples were taken, the stir-bar remained stirring with the samples taking 30 seconds to be withdrawn from the reactor. The retentate samples were withdrawn using a pipette and a long tip reaching in from the entry port of the stir-cell reactor. After each sample, 600 μL of buffer (without RNA) was added back into the stir-cell. After the last sample, the remaining retentate was removed and the stir-cell reactor was taken apart and washed with Isoclean. The membrane was saved, put in plastic wrap and allowed to dry.

From each permeate and retentate sample, 50 μL was withdrawn and concentrated in the speed-vacuum concentrator (Speed Vac® Plus SC110A Savant Instruments Inc; Farmingdale, NY). The resulting pellets obtained through the dewatering of the sample were resuspended with 20 μL of deionized water and 10 μL of 75 % formamide. An analytical electrophoresis gel was made similar to preparative gel described in section 2.2.2.1. The analytical gel included 25 mL of 20 % acrylamide solution, 150 μL of APS, and 50 μL of TEMED. The gel assembly was made the same way, except a 0.75 mm spacer was used and a 10-lane gel comb was used. Again the gel was run until the bromophenol blue dye migrated downward to within about 4 inches of the bottom of the gel. The plates were carefully removed and, in addition to the plastic wrap, a piece of gel paper was also placed on the opposite side of the plastic wrap. This was then placed in a gel dryer (BioRad Model 583 Gel Dryer; Richmond, CA) for 2 hours at 80° C and allowed to dry and cool for an additional hour.

The dried gel and membrane were placed in an exposure cassette (Molecular Dynamics; Sunnyvale, CA) for periods ranging between 1-10 hours, depending on the amount of radioactivity remaining on the gel. After exposure, the plate was then

visualized and quantified using a phosphorescent imager system (Molecular Dynamics; Sunnyvale, CA). The imager system displays an image of the gel on the computer monitor, which then can be manipulated. One such manipulation was to quantify the bands located on the gel by drawing regions around the bands. These regions can then be integrated to give a volume amount, which simply stated, is the pixel counts located in the region. These values are proportional to concentration values in the samples. From these values, a relative concentration of the retentate and permeate can be found and used to determine the rejection coefficient of the labeled 12-mer RNA transcript. Figure 2.4 illustrates a radioactive image of a typical electrophoresis gel showing the permeate and retentate samples which were used to obtain the data to determine the

$$R = 1 - \frac{C_P}{C_R}$$

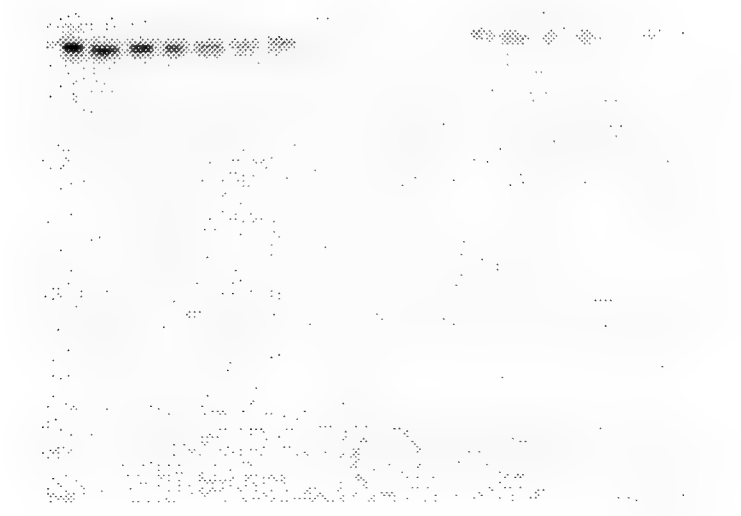


Figure 2.4 Image of a typical gel showing the radioactively labeled RNA 12-mer in the permeate and retentate samples. The band on the far left is the retentate at 0 time point. The next band is the 1 hour permeate sample followed by 1 hour retentate sample. This continues until the far right bands which is permeate and retentate 6 hours. The circle at the base of the image is the membrane.

concentrations of the permeate and retentate with time. The equation below was used to determine the rejection coefficient, R, with C_P being the concentration in the permeate and C_R being the concentration in the retentate (28).

2.2.3.2 Transcription Reactions

The transcription reactions were done to simulate actual transcription reactions taking place and how the transcription materials affected the membrane transmission of RNA. These materials included the agarose beads containing the DNA template, polymerase, unlabeled NTPs, and the transcription buffer described in Section 2.2.2.1. The transcription materials were inserted into the stir-cell bioreactor containing the YM100 membrane. With the addition of polymerase, the reaction immediately begins. The experiments contained all the materials for the production of RNA. The procedure as described in section 2.2.3.1 was followed with the exception of first letting the beads settle so that a clean retentate sample could be taken without removing beads from the bioreactor. Once again 10 μL of radioactively labeled RNA (12-mer) was added to the reactor with the transcription materials and a concentration of 3.34 μM. This was the only radioactive material located in the reactor. When samples were visualized, this labeled RNA was the only material quantified. The experiments were designed to simulate an actual reaction, so RNA was being produced in the stir-cell but this new RNA was non-radioactive and thus was not quantified. After each reaction, the remaining retentate was removed from the stir-cell, and then two washes were done with water to assure the removal of all beads from the reactor.

2.3 Results and Discussion

2.3.1 UU Hairpin (12-mer) Control and Transcription Results

The complete results of the RNA ultrafiltration studies are given in the following figures. The first set of reactions was to study transmission of the UU hairpin RNA molecule (12-mer) in buffer. The buffer experiments contained no beads or any of the materials present during a transcription reaction. In addition, another set of experiments was done to study the transmission of the labeled 12-mer when the transcription mixture was present in the reactor. The buffer experiments were conducted to determine a baseline for transmission of 12-mer RNA and to validate the procedures. With the baseline determined, experiments with the transcription mixture were done to determine RNA transmission when reaction components were added to the reactor. In Figure 2.5, the rejection coefficient is shown as a function of time for

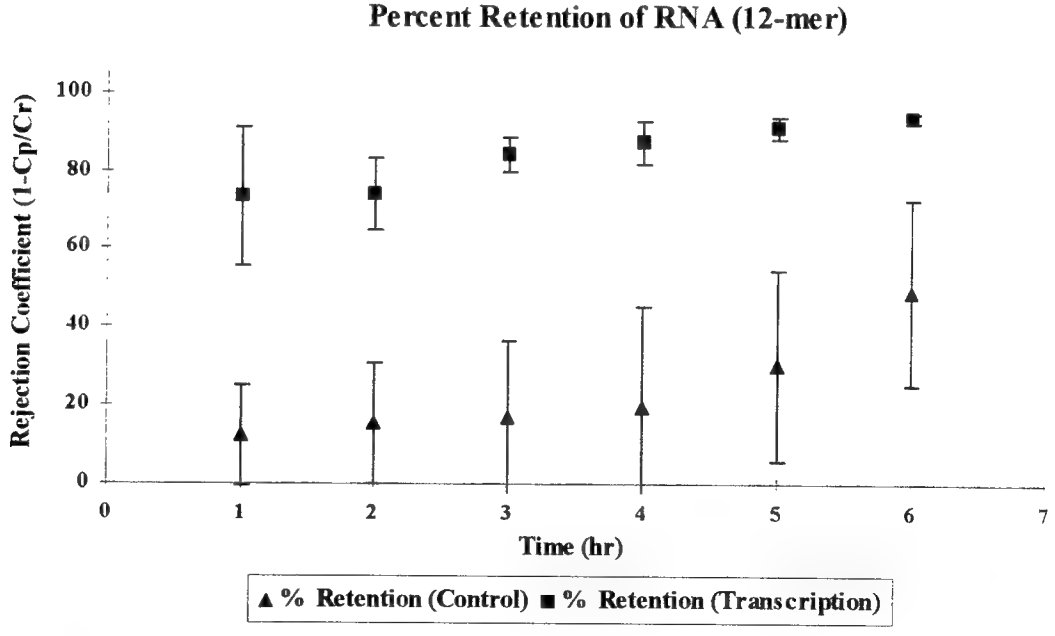


Figure 2.5 Transmission of RNA through the membrane over time showing that the transcription reaction mixture reduces the transmission of RNA compared to the buffer experiments. The error bars indicate plus and minus one standard deviation from the five of multiple experiments.

the buffer and transcription experiments. The retention of RNA is seen in both cases to increase with time, indicating that membrane fouling was occurring. In the buffer (control) experiments, the retention is approximately 10% initially, indicating that most of the RNA is transmitted through the membrane, whereas the initial retention of RNA is approximately 75% for the reaction mixture, indicating that most of the RNA is retained by the membrane.

Fouling in the membrane is a typical problem found in any membrane separation process. After each experiment, the membrane was removed and dried. When the gel was imaged the membrane was also imaged, thus enabling a visual indication of whether RNA was fouling the membrane. In both instances, buffer and transcription, radioactive counts (12-mer RNA) remained on the membrane, verifying that fouling was occurring. This fouling is greater in the transcription experiments. The amount of RNA left on the membrane in these experiments can be partly attributed to beads embedding on the top membrane surface thus blocking the pores of the membrane. These beads are porous and have the potential to adsorb RNA. By visualizing the membrane in both cases it is clear that the agarose beads which contained the DNA template were causing some of this fouling. Figure 2.6 shows an image of membranes taken from one buffer and two transcription experiments. The beads are porous structures that are an average of 50 microns in diameter (average measurement from the company). After transcription experiments, the beads were removed from the bioreactor and then water was added to the reactor to remove the remaining beads. Invariably when the reactor was taken apart and the membrane removed, some beads remained on the membrane. The beads were not chemically

attached to the membrane; when water was added to the surface of the membrane the beads could be removed, but after a six-hour reaction the beads became embedded in the membrane. This term embedded indicates that the beads were layered on the surface of the membrane. This could be caused by the suction of the syringe when permeate samples were drawn through the membrane. Once these beads are embedded into the membrane they remain there for the rest of the reaction. The fact that the stir bar within the stir-cell is unable to continuously remove these beads from the membrane is difficult to comprehend because the purpose of such a stir bar is to remove the concentration polarization layer. Perhaps the many hydrogen bonds located on the regenerated cellulose membrane are enough to attract the agarose beads and thus they become embedded or adsorbed to the surface of the membrane, with the stirring action unable to dislodge them. Although these beads do not become chemically bound to the membrane, the two species have a mutual affinity for one another since agarose and regenerated cellulose are both high in hydroxyl groups which promote hydrogen bonding.

Figure 2.6: Radioactive images of three membranes from a buffer (left) and transcription reaction (middle and right). The dark spots represent radioactive counts. The middle membrane clearly shows the spherical dots of radioactive counts which indicate the beads remain on the surface of the membrane. The right membrane shows considerably more fouling. There is no significant difference between the middle and right membranes.

If the beads collect at the surface of the membrane, the beads could act as an additional sieve. The beads are porous and, with enough beads sedimenting onto the membrane, they could act as a barrier to the transmission of RNA. The labeled RNA could also be adsorbed by the beads. With the transmission decreasing with time when the transcription materials are present, then over time more labeled RNA is being adsorbed by the beads and is no longer being transmitted.

In addition to the rejection coefficient, concentration profiles of the labeled RNA in the retentate and permeate under buffer and transcription conditions were plotted (Figures 2.7 and 2.8). The ideal profile is shown in both of these figures for

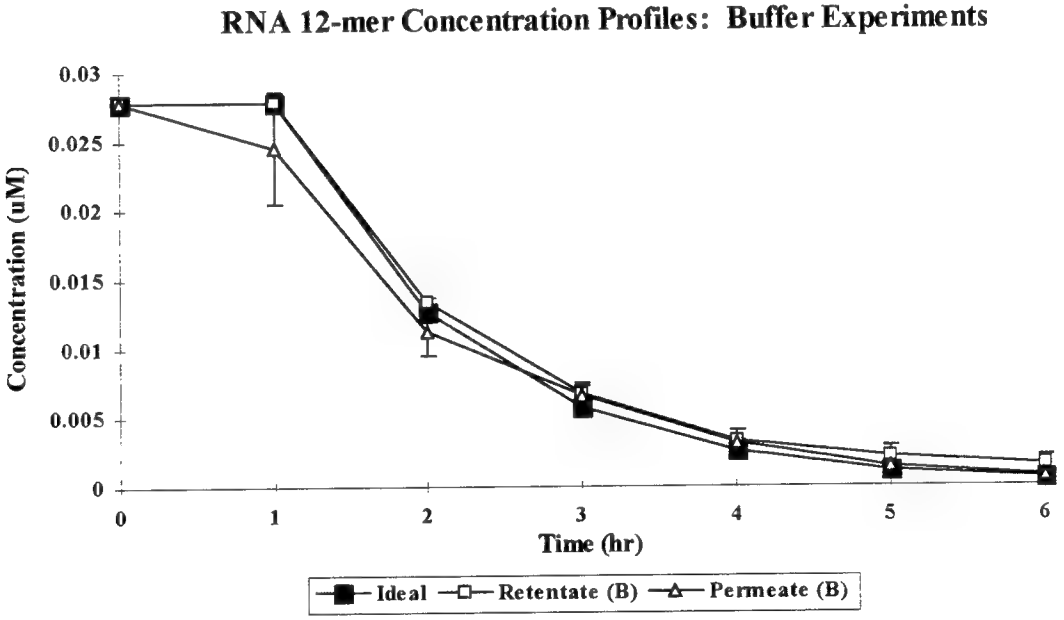


Figure 2.7: Concentration of RNA 12-mer in retentate and permeate as a function of time in the buffer experiments compared to an ideal case. The ideal case is determined by assuming that the membrane is ideal (100% Transmission). The error bars indicate plus or minus one standard deviation from the mean values of multiple experiments.

comparison, indicating the profile which would be seen if the membrane permitted 100% transmission. From the retentate and permeate profiles, it is seen that in the buffer experiments the 12-mer follows the ideal profile meaning that very little rejection and fouling is occurring. This should be the case since the membrane has a MWCO of 100,000 daltons, whereas the 12-mer has a molecular weight of 4084.2. When these profiles are produced for the transcription experiments, the 12-mer is mostly retained and very little of it is transmitted through the membrane. Since the buffer experiments followed closely to the ideal case, fouling of the membrane by RNA molecules alone is probably not the reason for the profiles seen in Figure 2.8.

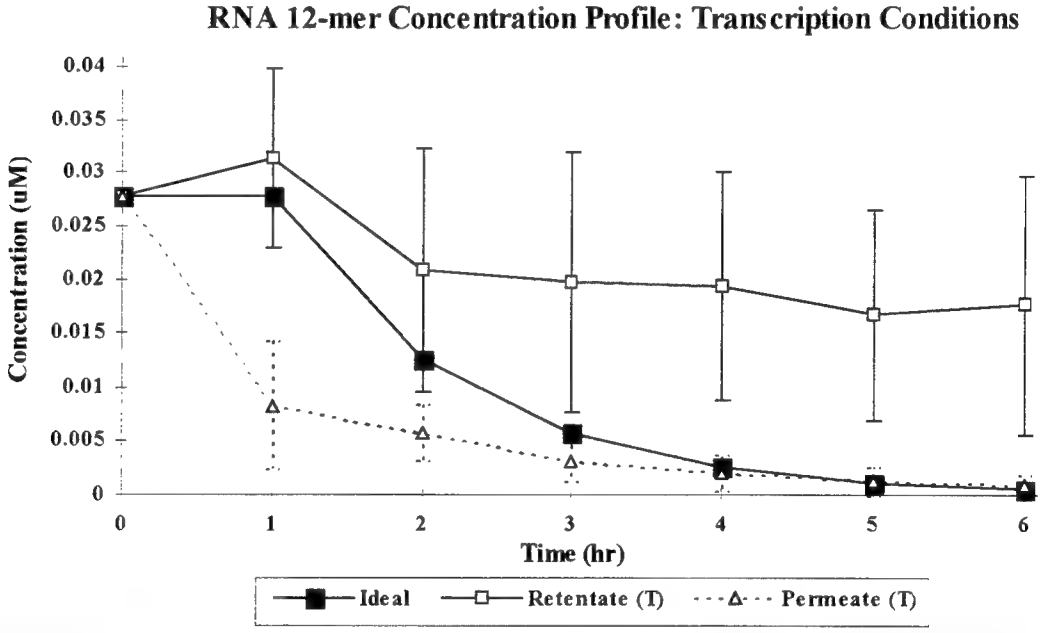


Figure 2.8: RNA 12-mer concentration versus time retentate and permeate in the transcription experiments compared to an ideal case. The ideal case is determined by assuming that the membrane is ideal (100% Transmission). The error bars indicate plus or minus one standard deviation from the mean values of multiple experiments.

The transcription mixture must provide additional cause for retention of RNA in the reactor. Possible culprits could be the beads and/or polymerase fouling the membrane, and the RNA molecules becoming associated with the beads and/or polymerase.

2.3.2 Pseudo-knot (28-mer) and Rigid-Rod (31-mer) Results

Further studies were accomplished using the pseudo-knot 28-mer RNA molecule. For this molecule, permeation experiments with both buffer (control) and transcription mixtures were performed to see if similar results were obtained in comparison to those found with 12-mer. The 28-mer studies show (Figure 2.9) trends

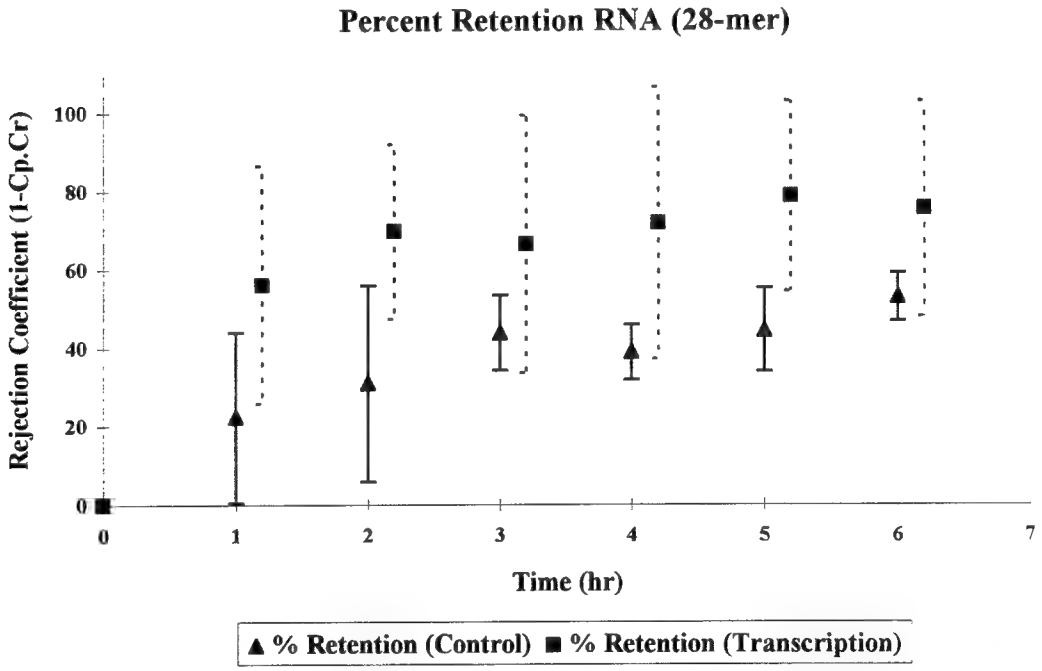


Figure 2.9 Transmission of 28-mer RNA through the membrane over time showing that the transcription reaction mixture reduces the transmission of RNA compared to the buffer experiments. The error bars indicate plus and minus one standard deviation from the mean of multiple experiments.

similar to those of the 12-mer, an increasing retention in buffer with time and a decreased transmission when the transcription mixture was added to the bioreactor. These trends can be seen in Figure 2.9. The trends observed from the 28-mer indicate that the difference between the buffer and transcription experiments is not as large as seen in the 12-mer studies. The membranes when viewed also showed similar trends with buffer membranes. The concentrations of the retentate and permeate were also plotted for the 28-mer. The results seen in Figures 2.10 and 2.11 show similar trends to those that the 12-mer showed. The transcription mixture causes the retention of RNA to be greater than when RNA is in only buffer.

In addition, buffer studies (Figure 2.12) were conducted on the rigid-rod 31-mer RNA molecule to compare with transmission of the 12-mer and 28-mer to see if

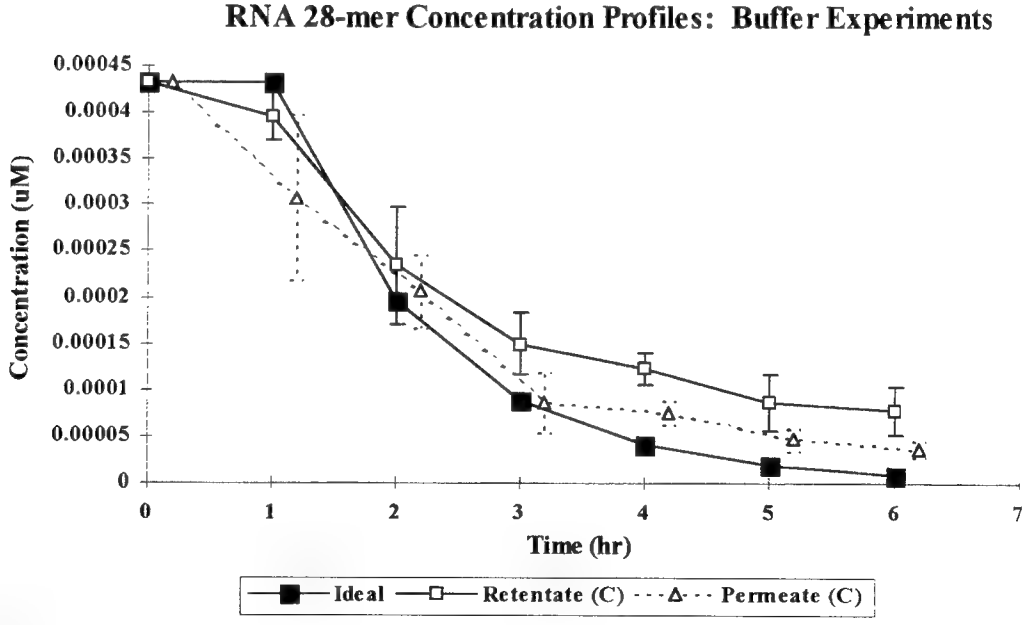


Figure 2.10: Concentration of RNA 12-mer in retentate and permeate as a function of time in the buffer experiments compared to an ideal case. The ideal case is determined by assuming that the membrane is ideal (100% Transmission). The error bars indicate plus and minus one standard deviation from the mean values of multiple experiments.

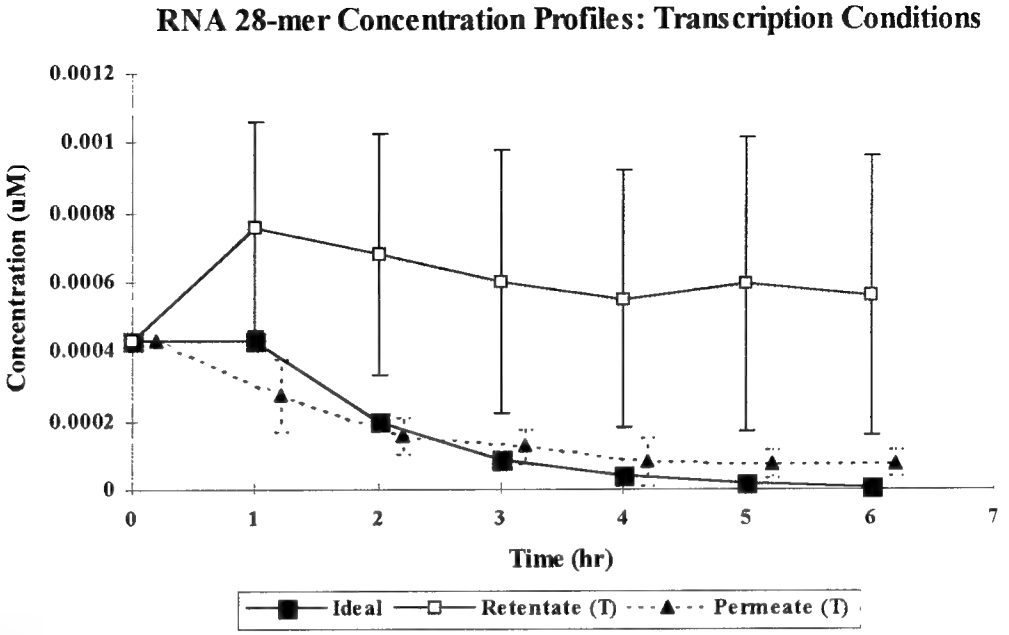


Figure 2.11: RNA 12-mer concentration versus time retentate and permeate in the transcription experiments compared to an ideal case. The ideal case is determined by assuming that the membrane is ideal (100% Transmission). The error bars indicate plus and minus one standard deviation from the mean values of multiple experiments.

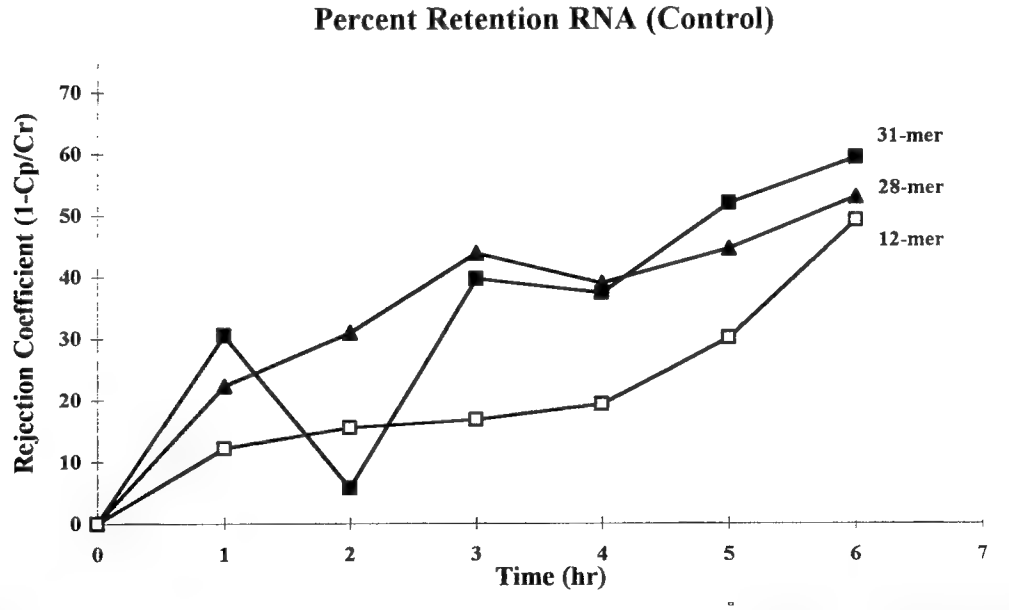


Figure 2.12: Comparison of rejection coefficients of three RNAs. Transmission of RNA was found to be more of a function of molecular weight then secondary structure of the molecule.

RNA secondary structure affects rejection. Figure 2.12 shows that the 28-mer with secondary structure and the 31-mer linear molecule have similar transmission characteristics (with the exception of one anomalous datum); both exhibit higher retention than the smaller 12-mer. Figure 2.13 shows the three RNA molecules studied in these experiments.

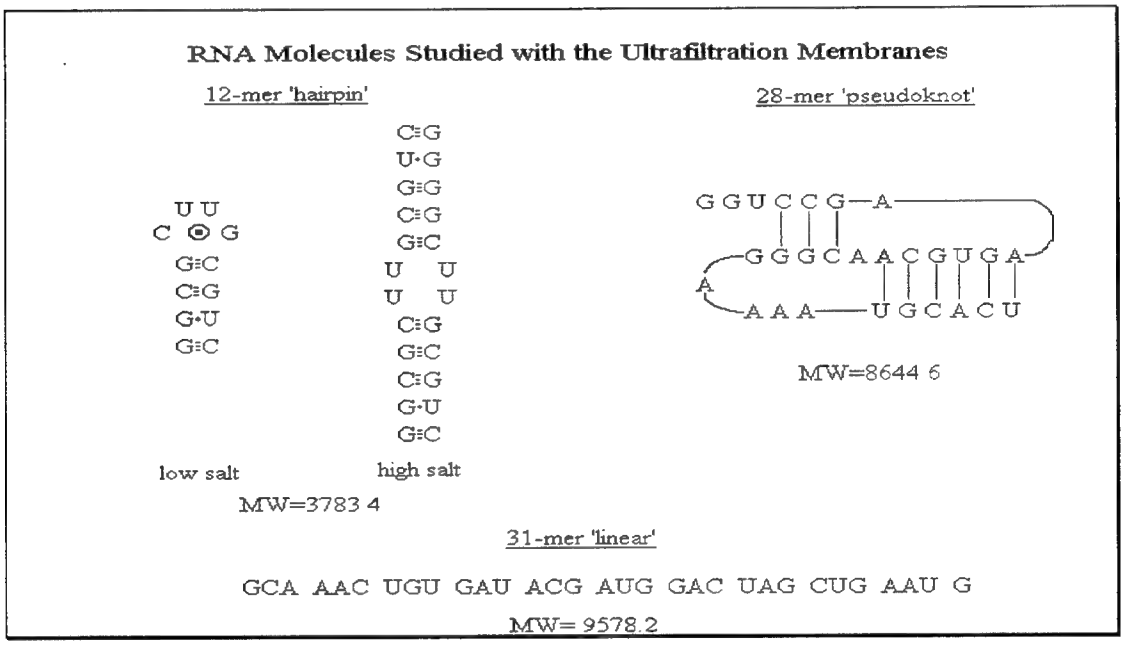


Figure 2.13: Diagram showing the secondary shapes of the RNA molecules studied.

2.3.3 Transmission of RNA Through Membranes of Different Porosities and Composition

Finally, comparisons of transmissions for different membrane pore sizes, as well as for different membrane materials, were done (Figure 2.14 and 2.15). YM30 and the YM100 membranes were studied using the 12-mer RNA to test the effects of the pore size of the membrane. The YM30 has a MWCO of 30,000 while the YM100

has a MWCO of 100,000. The YM series from Amicon is composed of advanced hydrophilic membrane with low non-specific protein binding properties. In addition,

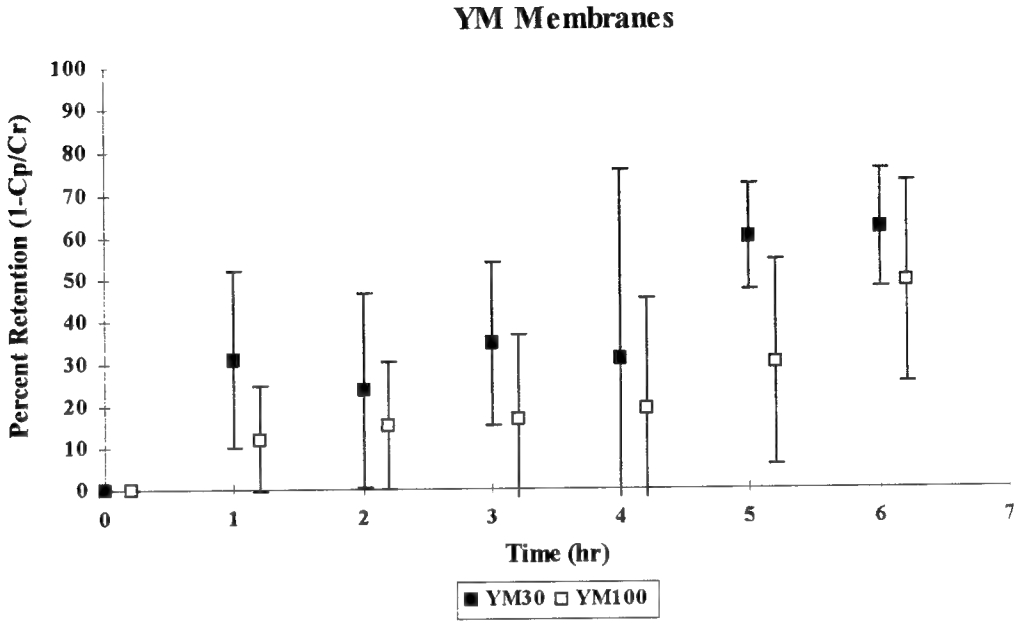


Figure 2.14: Rejection of 12-mer RNA by YM membranes of varying MWCO

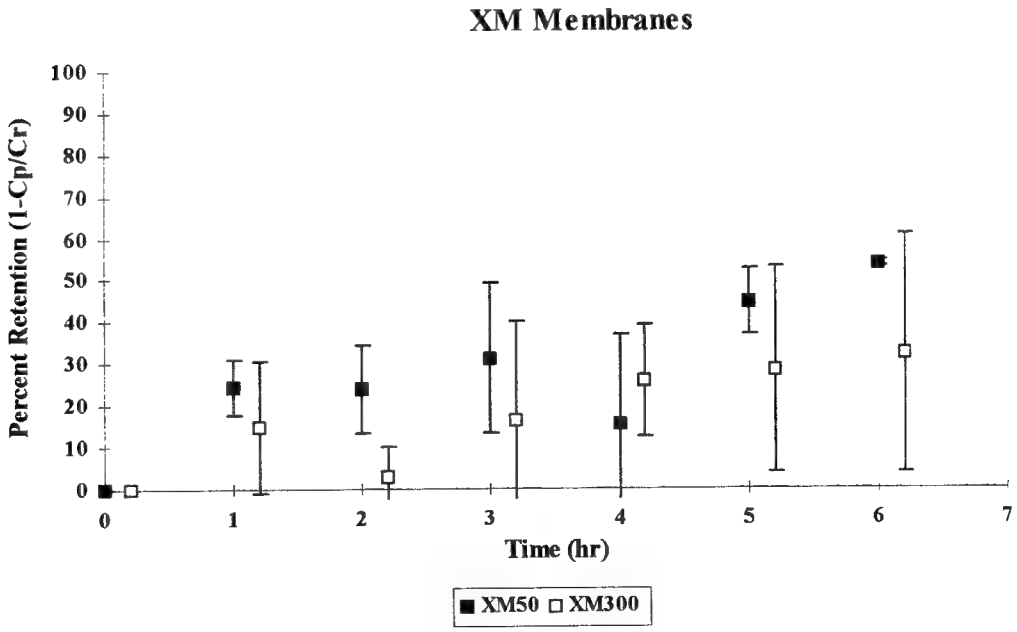


Figure 2.15: Rejection of 12-mer RNA by XM membranes of varying MWCO

XM50 and XM300 membranes were studied as well, with a MWCO of 50,000 and 300,000 respectively. The XM series of membranes are characterized as being non-ionic and moderately hydrophilic.

The data indicate that the transmission decreases with a lower molecular weight cut-off. This is an expected result. The results also indicate that by increasing the MWCO to 300,000 not much is gained in transmission. Furthermore, using a membrane of this size, higher molecular weight compounds such as polymerase will show an increase in transmission since T7 polymerase is a 98 kD protein. These studies indicate that the YM100 membrane is the best membrane of those tested to use for this process. Using a smaller membrane such as the YM30 or XM50 would satisfy the conditions of separating the RNA and retaining the polymerase, but a decrease in RNA transmission would be the result. The YM100 is still small enough to retain about 90 % of the polymerase and, since separation is the goal, this is the membrane of choice (29).

The studies above have shown the effectiveness of using an ultrafilitration membrane for the separation of RNA from transcription materials. A concern is the decrease in transmission when the transcription reaction mixture is included in the stir-cell. This decrease in the amount of RNA transmitted needs to be explained and overcome so that the process can become more effective.

2.4 Conclusions

The purpose of these studies was to determine the transmission of RNA through an ultrafiltration membrane and to determine the effects of the transcription reaction on transmission. RNA transmission in buffer is about 80%. When the transcription reaction is taking place in the reactor then the transmission drops to about 20%. This trend is seen with two RNA molecules, the 12-mer and 28-mer. The reason for this drop in transmission is that the membranes after a transcription reaction show considerable fouling. Another study was done using a 31-mer and this study compared the transmission of all three RNA molecules and determined that secondary structure of RNA does not cause differences in transmission. The molecular weight is the determining factor for transmission. Finally studies were done to determine if the YM100 was the optimal membrane to use for the retention of polymerase and template and transmission of RNA. The studies comfirm that the YM100 is the membrane of choice although a larger pore membrane would transmit more RNA a greater amount of polymerase would be lost.

CHAPTER III

CHARACTERIZATION OF TRANSCRIPTION MATERIALS IN THE STIR-CELL

3.1 Objective

The objective of these characterization studies was to determine the feasibility of using a bioreactor for continuous production of RNA. Previous studies indicated that the use of a stir-cell bioreactor is a viable alternative for the production of RNA (20). Further studies are needed to fully understand how the transcription materials, as well as the transcription reaction, behave in a stir-cell set-up before further scale-up is attempted. Additionally, before scale-up was studied, an in-depth study needed to be accomplished to try to understand the effects seen in Chapter II, and to determine if the transcription reactants were affected by the stir-cell. The effects seen in Chapter II included the possibility of the agarose beads adsorbing RNA and these beads adhering to the surface of the membrane during the reaction. The areas studied in this chapter included experiments to determine if the agarose beads adsorb NTPs and/or RNA, whether the polymerase was deactivated by the stir-bar, determination of the effects of the membrane on the reaction, and determining the durability of the reaction

materials over long periods of time in the stir-cell. After characterizing these effects, the semi-continuous bioreactor was used to determine the rate of RNA production over time for comparison with a batch reactor.

3.2 Materials and Method

3.2.1 Agarose Bead Adsorption of NTPs and RNA

A study was performed to determine if the agarose beads were adsorbing NTPs and, if so, how the concentration of nucleoside tri-phosphates affect the amount of material adsorbed by the agarose beads. This study was performed because the research in Chapter II showed that RNA was adsorbed by the beads. If RNA could be adsorbed, then the potential exists for the NTPs to adsorb as well, and such adsorption would cause a decrease in available NTPs that are needed for the reaction to continue. To test this possibility, a standard was created by diluting the stock solution of radioactively-labeled uridine triphosphate ([α-^{32}P]-UTP, 800 Curies/millimole, DuPont/NEN; Wilmington, DE) which was at a concentration of 10 μCi/μL on the shipping date. This date is important in that the concentration is exactly 10 μCi/μL at that time, and any date before or after must be accounted for because the concentration changes as ^{32}P decays with a half life of 14.3 days (30). The amount was defined in μCi and, from the dilutions, a standard was created ranging from 1 x 10^{-1} to 1 x 10^{-7} μCi. These amounts were blotted onto filter paper and then exposed on a phosphor screen for one hour. The plate was developed on a PhosphorImager

(Molecular Dynamics; Sunnyvale, CA), and the resulting blots were circled and quantified using the image analysis software. The data from this analysis give a volume integration value which is related to concentration. These volume integration values were plotted against the corresponding amounts (μCi), and the resulting calibration line was used in the ensuing experiments (Figure 3.1).

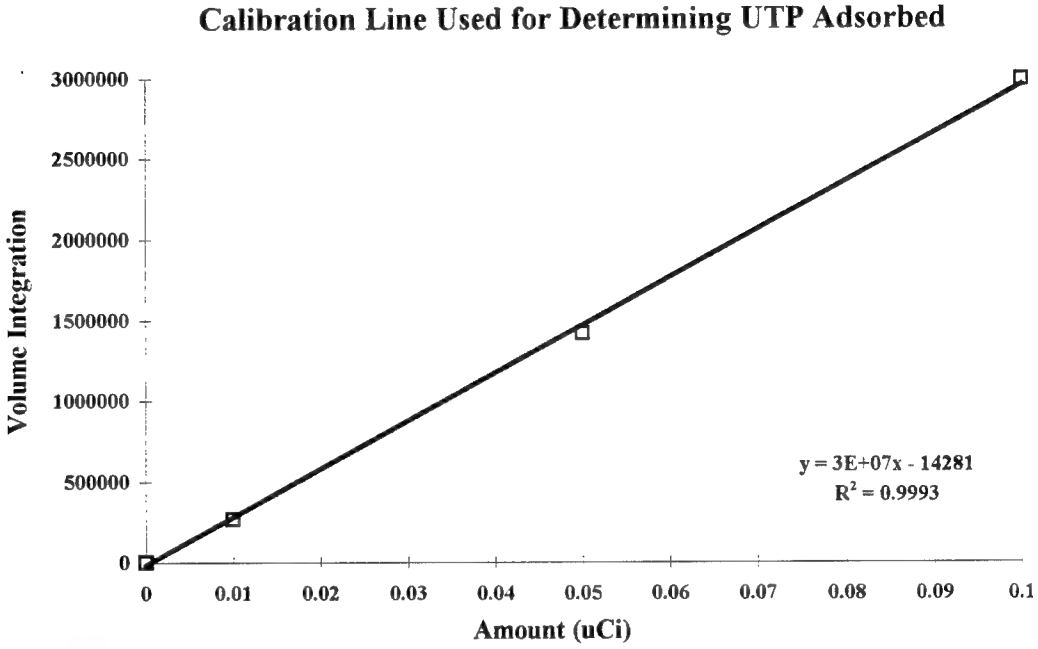


Figure 3.1: Sample calibration line used for the NTP adsorption studies. The volume integration values are arbitrary values set by the software.

From the stock solution of radioactively-labeled uridine triphosphate (10 μCi/μL) dilutions were made, giving concentrations of 10, 7, 3, 1, 0.7, 0.3, 0.1 μCi/ μL. These amounts were added to a 100 μL reaction volume which included 20 μL 50 % v/v biotin-DNA-streptavidin coated agarose beads (Pierce Immunochemicals; Rockford, IL) solution, an amount of total NTPs proportional (10, 7, 3, 1, 0.7, 0.3, 0.1 μL of a 10 X solution of NTPs) to the amount of labeled uridine triphosphate, and RNAse free water. No polymerase or reaction buffer was present in the reaction.

This mixture was allowed to mix for one or four hours and then stopped by spinning the beads down and removing 80 μL of the supernatant. From the supernatant, 10 μL was removed, blotted onto filter paper, and allowed to expose on the phosphor plate for one hour. The plate was developed on the PhosphorImager, and the resulting blots were quantified. Each datum gives a volume integration amount that is then corrected for the dilution and compared to the calibration standard to obtain an amount in μCi. This amount is how much radioactively-labeled uridine triphosphate was not adsorbed by the beads.

A similar set of experiments was done to determine if RNA was adsorbed by the agarose beads. The mechanism of binding of RNA is the adsorption of RNA into the pores of the RNA or the hybridization of the RNA molecules to the DNA template. The first set of experiments was done by adding purified 12-mer RNA (.0601 μg/μL) in different volume amounts to suspensions of beads. For a final volume of 100 μL, 20 μL 50 % v/v biotin-DNA-streptavidin (1 μM DNA) coated agarose bead solution was added to RNAse free water along with the purified RNA. The solution was allowed to mix for one hour, and then the beads were centrifuged down and 80 μL of supernatant was removed. The supernatant was mixed with 320 μL water to obtain a final volume that was needed for the diode array spectrophotometer (Hewlett-Packard; Palo Alto, CA) to obtain an absorbance reading at 260 nm. From the absorbance reading a concentration was obtained by using the molar absorptivity and the molecular weight. Using the Beer-Lambert Law, $A = \varepsilon b c$, where ε, the molar absorptivity, b, the cell length (1.0 cm), and c, the concentration

in molarity. The molar absorptivity for nucleic acids is found by determining the sequence of the RNA molecule and counting the individual bases. The formula for the molar absorptivity, ε (per Molarity), at 260 nm, 25 °C, and pH 7.0 is

$$(A * 1.54E04) + (U * 1.17E04) + (C * 0.73E04) + (G * 0.88E04) = \varepsilon$$

The A, U, C, and G are the number of each of the bases in the sequence (31). The result is 114100 / cm*M for the RNA used, which has a molecular weight of 3783.4 g/mol.

A second experimental procedure was followed using radioactive 12-mer RNA produced in a transcription reaction using [α-^{32}P]-UTP. A standard (Figure 3.2) was created by measuring volume amounts of purified labeled RNA ranging from 5 to 50 μL. A volume measurement was taken since it was impossible to obtain a μCi/μL

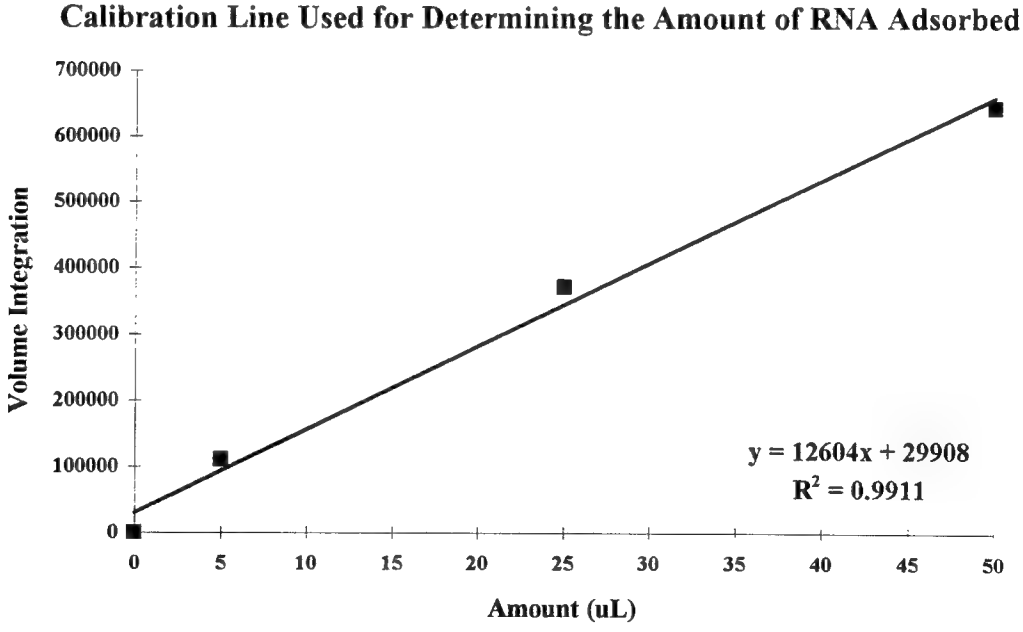


Figure 3.2: Calibration line used for determining the amount of RNA adsorbed by the agarose beads. Amount is the volume of RNA used in solution.

value for how much RNA was radioactively labeled because of the unknown amount of activity located specifically in the RNA molecules. These amounts were blotted onto filter paper and then exposed on a phosphor screen for one hour. The resulting blots were circled and quantified using ImageQuant software giving a volume integration value. These volume integrated values were plotted against their respective volume amounts, and the resulting linear regression was used as a standard in the following experiments.

To a 100 μL reaction volume, 20 μL 50 % v/v biotin-DNA-streptavidin coated agarose bead solution, RNAse free water and labeled RNA were added (20, 10, 5, and 1.0 μL). This mixture was allowed to mix for one hour and then stopped by spinning the beads down using a centrifuge and removing 80 μL of the supernatant. From the supernatant, 10 μL was removed, blotted onto filter paper, and allowed to expose on the phosphor plate for one hour. The plate was developed on the PhosphorImager and the resulting blots were quantified. Each datum that is given as a volume integration amount is corrected for the dilution and then compared to the standard to obtain an amount in μL. This amount is how much radioactively labeled RNA was not adsorbed by the beads.

3.2.2 T7 Polymerase Deactivation

To better understand the behavior of the polymerase within the stir-cell, experiments were conducted to determine the stability of the polymerase within the stir-cell. A series of experiments was performed to determine the stability after a three

hour transcription reaction, as was an experiment to test for the rate of deactivation. The three-hour experiment was designed to determine if the polymerase deactivated over a long period of time, and from these results a rate-of-deactivation test was accomplished to determine how quickly this deactivation occurred. A 1000 μL non-radioactive transcription reaction was set up in the stir-cell containing the T7 transcription buffer, NTPs, beads containing 1 μM DNA template, 0.023 mg/mL T7 RNA polymerase, and d_AH_2O for 3 hours. At 30 minute timepoints, 59 μL was removed from the retentate within the stir-cell and transferred to a 100 μL batch reaction which contained the materials described above, except no polymerase, and which contained radioactively-labeled uridine triphosphate. No beads were transferred to the batch reaction vessel because before a timepoint was taken, the beads were allowed to settle. The purpose of this experiment was to determine how long the polymerase remained active in the stir-cell. By seeding the polymerase in the stir-cell at time zero, and taking aliquots out of the stir-cell at various times and placing these aliquots which contained polymerase into a batch reaction mixture with no other polymerase, one can determine the effects of the stirring action and other effects of the stir-cell on the polymerase by determining how well it still transcribes.

In addition to taking timepoints every 30 minutes, another set of experiments as done to determine when the polymerase deactivated or the rate of deactivation. In these experiments, timepoints were taken at 0, 5, 15, 30, 45, and 90 minutes, and the same procedure as described above was followed. At the specified timepoint, a 59 μL sample was removed from the stir-cell reaction and placed in an otherwise polymerase-

free batch reaction. These reactions were placed on a rotary mixer at 37° C. After four hours, the beads were spun down and the supernatant was removed and loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate.

Three controls were performed to compare the results found in the deactivation studies. The first control was accomplished by performing a standard 100 μL batch transcription reaction which contained the T7 transcription buffer, NTPs, beads containing 1 μM DNA template, 0.023 mg/mL T7 RNA polymerase, and d_AH_2O. This control was performed to compare a standard transcription reaction with the polymerase deactivation studies to determine if any differences exist between fresh polymerase and polymerase that has been first seeded in the stir-cell. The second control was performed in a similar manner as described above in the polymerase deactivation studies except 59 μL samples were removed from a 1000 μL batch transcription reaction and placed in a 100 μL batch reaction. The purpose of such a control was to determine if the impeller shear in the stir-cell was affecting the polymerase activity. The final control was also done to determine if the polymerase was being affected by the impeller. The possibility exists that the stirring motion caused by the impeller could be causing shear effects that potentially could affect the structure of polymerase. Since this structure is important in controlling the transcription of RNA from DNA, any affect on the polymerase due to shear is a potential problem for any scale-up procedure. A 1000 μL non-radioactive

transcription reaction was performed in the stir-cell with the stir-bar **stationary**. At 1,2, and 3 hours, 59 μL of retentate was removed from the stir-cell and placed in a 100 μL batch reaction. These batch reactions were allowed to run for 4 hours on a rotary mixture at 37 degrees Celsius, then the beads were centrifuged, and the supernatant was removed. These samples were loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was scanned on the PhosphorImager.

3.2.3 Membrane Compatibility

To further characterize the production of RNA in the stir-cell, the membrane had to be tested with the transcription mixture to assure that the reaction was not affected by the membrane. A 1000 μL transcription reaction was performed in an Amicon Centricon-3 concentrator (Amicon; Beverly, MA). The reasons for such a configuration were to simulate a stir-cell with a membrane but to follow a batch transcription method using a rotated vessel rather than a stir-bar. The membrane was a YM-3 with a MWCO of 3000 but in composition similar to the YM-100. The Centricon-3 was cleaned by filling it with a 70% ethanol solution and centrifuging it in the Superspeed centrifuge (DuPont Sorvall® RC 5C Plus) for 1 hour at 6000 rpm, then removing ethanol and repeating the procedure with RNA elution buffer containing 10 mM Tris and 1 mM EDTA. The reaction was set up on a rotary mixture to stimulate the batch method of RNA production. From the concentrator, which served as the

reaction vessel, 10 μL samples were removed at 0, 30, 60, 90, 120, and 180 minutes These samples were loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was scanned on the PhosphorImager.

3.2.4 Agarose Bead Loss

After doing a series of experiments, it became apparent that beads are lost in the stir-cell, which includes beads sticking to the surface of the stir-cell and membrane. To determine the amount of beads lost during a reaction, a 240 μL 50% v/v biotin-DNA-strepavidin coated agarose bead solution was diluted and pipeted into a 0.1 mm deep slide hemacytometer to count how many beads were in a typical transcription reaction. The bead size distribution is unknown although the average diameter of the beads is given by the manufacturer as 50 microns. A non-radioactive stir-cell transcription reaction (1000 μL) was performed with 200 μL if retentate taken every 30 minutes for up to 3 hours. After 3 hours, the beads were removed with a pipette, diluted, and counted. The hemacytometer was then used to determine how many beads were lost during the reaction.

3.2.5 RNA Degradation and Bead Durability

A study was performed to determine the possibility of RNA degradation over time. As the NTPs were depleted, the amount of pyrophosphate contained in the

reaction increased. This potentially caused the polymerase to go backwards and degrade RNA back into NTPs. To test this, a 1000 μL non-radioactive standard transcription reaction was setup in the stir-cell and the reaction ran for 4 hours. At the 4 hour point, 100 μL of a radioactive reaction mixture that contained 12-mer and accompanying aborts was inserted into the bioreactor. At 5, 6, 7, and 26 hours, 20 μL of sample from the retentate side of the membrane was removed. No solution was added back to the reactor and to each sample 5 μL of 75% formamide was added and loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager.

An integral aspect of using an immobilized-template reactor is that the beads containing the DNA template must be sturdy enough to be used over a long period of stirring. If the template were denatured over time, the beads would have to be replaced, adding cost to the process. To determine the stability of the beads, a series of experiments was done with the agarose beads within the bioreactor for extended periods of time. After a transcription reaction, the remaining solution (without the beads) was removed and replaced with 2.1 mL d_AH_2O and allowed to stir uninterrupted for 12 hours. The water was then removed through the membrane using the syringe. After removal of the water, a 1000 μL transcription reaction which included buffer, NTPs, and polymerase was begun. At 1, 2, and 3 hours, 100 μL retentate and 100 μL permeate samples were taken. No solution was added back to the reactor and, from each sample solution, 20 μL was removed. This 20 μL sample

was added to 5 μL of 75% formamide and loaded onto 20% (19:1 crosslinking) denaturing (8 M urea) polyacrylamide gel for electrophoresis (600 volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphoImager.

Additionally, a transcription reaction was performed after 24 hours of continuous stirring. The procedure in the above paragraph was followed, except 5, 10, 15, 30, 45, 60, 90, and 120 minute timepoints were taken to get a better understanding of the rate of transcription after the beads had been continuously stirred over a long period of time.

3.3 Results and Discussion

3.3.1 Agarose Bead Adsorption of NTPs and RNA

A study was performed in an effort to determine a possible reason for the decrease in RNA production found in the stir-cell. One hypothesis is that within the stir-cell the beads adhere to the membrane after a permeate sample is drawn with the syringe. These beads remain on the membrane despite the stirring action caused by the stir-bar. These beads are no longer in suspension in the transcription solution. Because the beads are porous and made of agarose that contain hydroxyl groups, the beads might be adsorbing the nucleoside tri-phosphates (NTPs). This hypothesis is reached partly due to the results obtained (Chapter II) showing that the membranes, when analyzed with the PhosphorImager, reveal radioactive counts and from the

visualization of the membrane showing an aggregated collections of beads. These studies determined that RNA was being adsorbed by the beads. If RNA could be adsorbed, then the potential exists for the NTPs to be adsorbed as well. Although these beads are also present in regular batch reactions, it is believed that the beads stay in suspension within the transcription solution so that the beads are continuously being washed by the solution and possibly an equilibrium state is reached. In the stir-cell, where the beads sediment onto the membrane, the beads are no longer continuously washed, and the NTPs that become adsorbed remain in the beads and are no longer available for transcription. This is another possible explanation for a reduced rate of RNA transcription over time, because the amount of available NTPs is reduced within the stir-cell. The significance is that, if scale-up is to be done, then the amount of NTPs being adsorbed could increase the cost of producing RNA. On the other hand, this retention of NTPs might be a positive in the stir-cell configuration, since reversibly adsorbed NTPs would be less likely to pass quickly through the membrane.

NTP adsorption by beads can be seen Figures 3.3 and 3.4 where adsorption isotherms developed from the data found in this study are plotted. The adsorption isotherms reveal that the more NTPs, specifically UTP, introduced to the beads the more the beads adsorb. The data also indicate that for longer time periods the amount of UTP adsorbed is increased. The range of μCi in the RNA transcription experiments is higher than normally used in a transcription reaction. Typically, a range of 1-2 μCi of labeled UTP is used. The trend seen in the Figures is important in that higher

amounts of labeled UTP are being adsorbed, so in a normal reaction, increasing the amount of NTPs causes the beads to adsorb more. This adsorption should

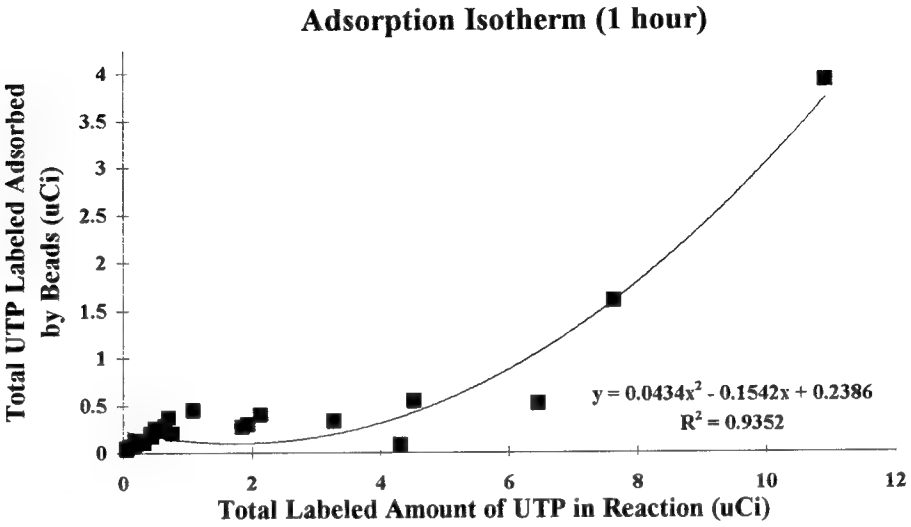


Figure 3.3: Adsorption Isotherm for the Agarose Beads and Nucleoside Triphosphates (UTP) for 1 hour. In this plot the reaction had not reached equilibrium.

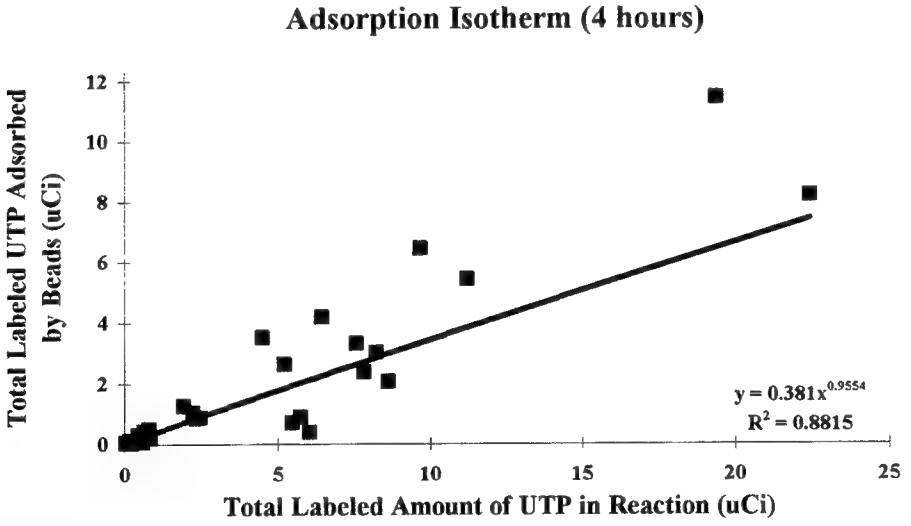


Figure 3.4: Adsorption Isotherm for Agarose Beads and Nucleoside Triphosphate (UTP) for 4 hours.

eventually reach an equilibrium. From the data above, it is impossible to distinguish equilibrium from a linear isotherm. Equilibrium assumes that the NTPs are adsorbing

to beads chemically rather than passing through. One positive note is that, in a continuous membrane bioreactor process, the NTPs would pass through the membrane very quickly, so the fact that the beads are adsorbing NTPs would increase the retention time of these reaction materials. Furthermore by using the equilibrium line, one can try to optimize the reaction with respect to NTPs

In addition to the NTP adsorption studies, an experiment was performed to determine the amount of RNA adsorbed by the beads. From Figure 2.6 in Chapter II it can be seen that radioactive counts remain on the membrane in the form of beads, indicating that RNA is potentially being adsorbed by the agarose beads. Since the only material that could be seen was the radioactive RNA, the conclusion was that RNA is reacting or adsorbing to the beads. Two studies were conducted to confirm this, a non-radioactive study and a radioactive study. The first study using the spectrophotometer indicated that less than 10 percent of the RNA that was put in the bead solution was adsorbed. This study was then followed up by using radioactively labeled RNA, and the results indicated that almost 80 percent of the RNA was adsorbed. The discrepancy is probably due to the fact that the spectrophotometer is not very accurate when dealing with small concentrations of RNA. A general conclusion that can be drawn is that RNA is probably adsorbed into the beads. Whether this amount is significant cannot be determined. Since these same beads are used in the batch reaction studies and these reactions have shown significant amounts of RNA being made the conclusion is that an equilibrium state is probably reached and that RNA is adsorbed but not significantly.

3.3.2 T7 Polymerase Deactivation

Another potential cause of decreased transcription potential in the stir-cell is that the polymerase is deactivating. The stirring action of the impeller could potentially cause the polymerase to denature. In the experiment to investigate this, the polymerase was first seeded into the reactor and then a portion of the transcription solution is removed from the stir-cell which contains polymerase after a period of time and placed in a batch transcription reaction. By removing this transcription solution at different times, it is hoped to get a profile of when the polymerase deactivates. This study indicates that the polymerase loses its transcription ability. The polymerase appears to initiate, but fails to elongate and complete transcription of the full length product, in this case the 12-mer. This leads to a possible conclusion that the polymerase's tertiary and quaternary structure could be affected and thus the conformational change needed to elongate is unable to occur. Figure 3.6 shows the gel of the RNA products made from polymerase, which had first been seeded in the reactor. The lane which is labeled control is a transcription reaction in which the polymerase is not seeded in the reactor. The RNA distribution is what is normally seen in a batch transcription reaction. The polymerase is fresh. The lane which is labeled beads signify a reaction in which no polymerase was present in the reaction. The reason for such a control is to make sure the aborts that are seen are not just NTPs spontaneously forming RNA transcripts. Figure 3.5 shows a typical RNA

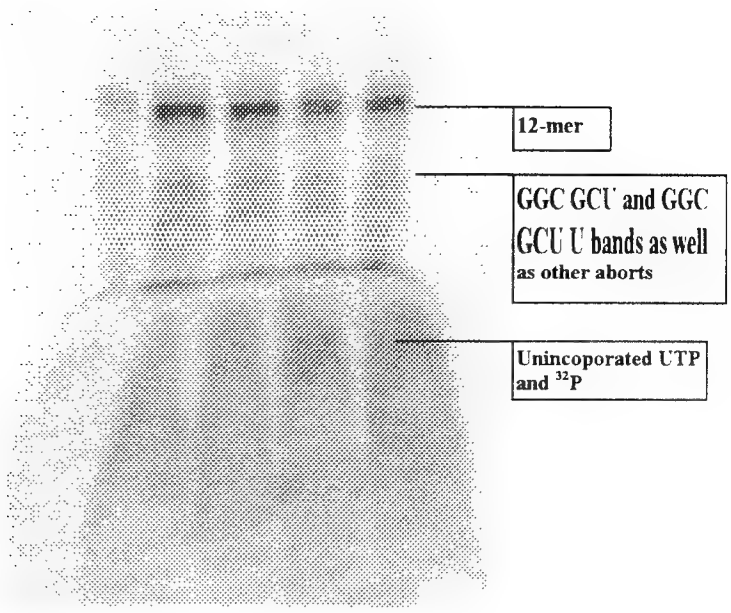


Figure 3.5: Electrophoretic gel showing the typical distribution of RNA made in a transcription reaction. The desired 12-mer is the dark band at the top. The bands below this band are the smaller aborts which are due to the polymerase dissociating from the DNA template before desired RNA product is made. The smear below the bands is unincorporated UTP, pyrophosphate, and waste material from the original radioactive UTP stock.

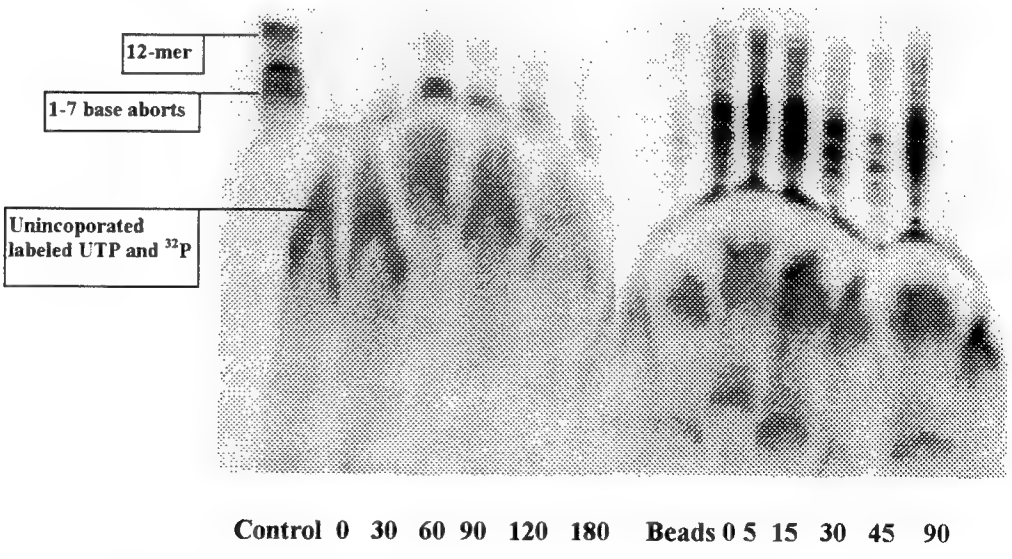


Figure 3.6: Electrophoretic gel showing the distribution of RNA when the polymerase was first seeded in the stir-cell then removed and placed in a batch transcription to determine polymerase activity. Notice how the distribution differs from that in Figure 3.5 in that the amount of aborted products in the 1 to 7-mer range is high and the amount of 12-mer made is minute. The numbers represent time in minutes. The bead lane represents a reaction with just beads, buffer and NTPs with no polymerase.

distribution of products for comparison. A graphical comparison of this RNA production distribution is shown in Figure 3.7, which gives the percent termination for each position in the message. The percent of abortive terminations, at a certain position in the 12-mer, is calculated as the amount of radioactive aborted product of a given length divided by the total radioactivity of products of that length or longer. Figure 3.7 denotes the percent of aborts that are terminated before the formation of the next bond (32). From this figure, an increase in the percentage of aborts as opposed to a typical reaction can be seen for the polymerase that was first seeded in the stir-cell. An interesting observation in Figure 3.6 is that the amount of aborts is

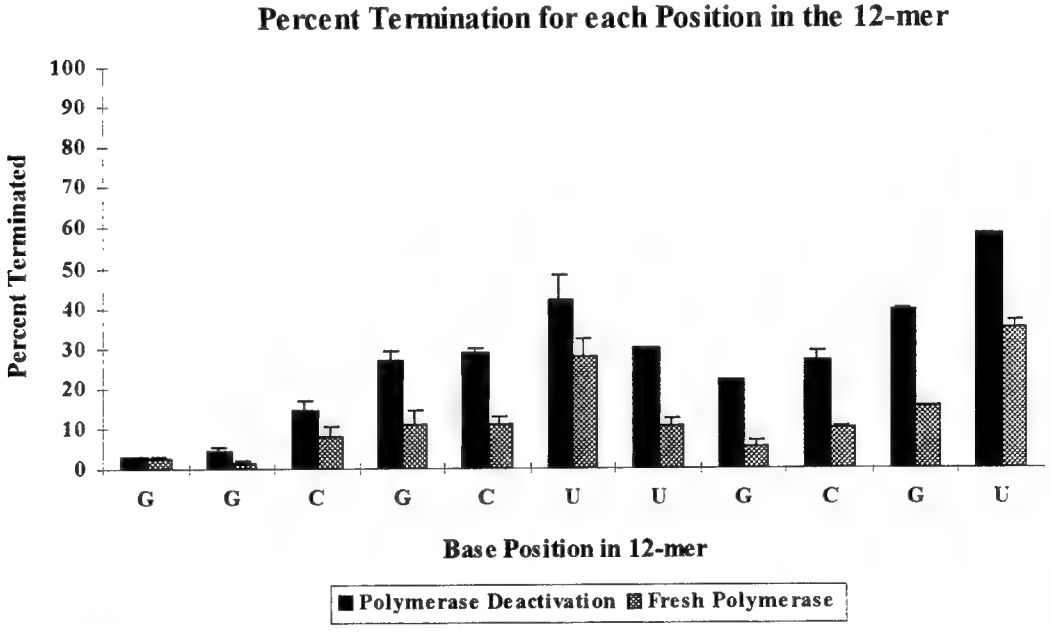


Figure 3.7: Graph showing the percent of abortive termination at each base in the 12-mer sequence. The fresh polymerase data come from Figures 3.5 and other similar data (not shown) while the polymerase deactivation data come from Figure 3.6 and additional deactivation studies (pictures not shown). The error bars represent one plus standard deviation.

constant as a function of time. One would expect the amount of aborts to increase and the amount of full length 12-mer to slowly decrease, with seeding time, with a corresponding increase in aborts. This is because the polymerase is spending more time in the reactor thus more total polymerase is being denatured.

A control was performed with polymerase being taken out of a batch reaction after specific time points and being placed in another batch reaction to determine if the polymerase remains active. In Figure 3.8 one can see the gel from this control experiment. From the image, one can see that significantly more aborts appear, but also one can see that full length product is formed in a minor amount. The conclusion is that the polymerase deactivates naturally with time in the reactor but significantly more with the impeller present.

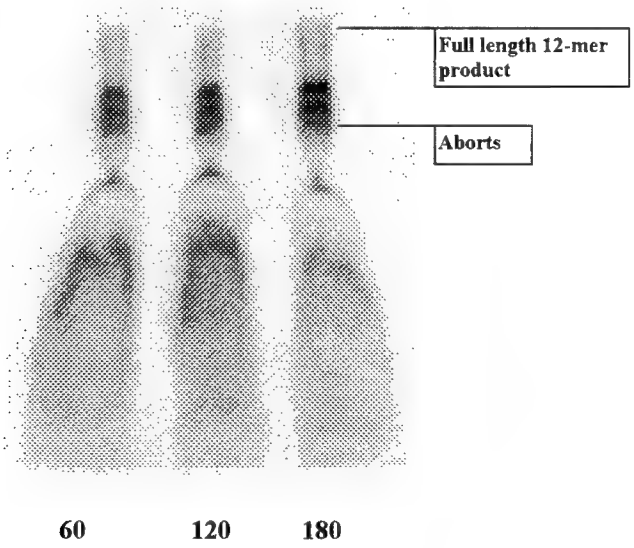


Figure 3.8: Gel of control experiment in which the polymerase was first seeded in a batch reactor and moved to another batch reaction. The image shows that aborts are still present in a greater amount compared to the typical transcription reaction but full-length 12-mer is also made.

From previous studies it has been speculated that impeller shear could be damaging the polymerase. To determine if this were true, a transcription reaction which included polymerase was started in the stir-cell. The stir-bar in the stir-cell was stationary and samples (containing polymerase) were removed at specific times. When the aliquots were removed and placed in a batch reaction, no transcription occurred. This observation supports the idea that polymerase remains near the template. With the template sedimenting to the surface of the membrane, the polymerase appears to be settling with the beads and template and is thus not free in solution. This could be expected if the ratio of DNA template to polymerase were high. In a typical reaction there exists 1 μM of DNA to .257 μM of T7 polymerase. There would be 4 DNA templates for every molecule of polymerase. If polymerase were at a higher concentration, then possibly the enzyme would exist in free solution. This result reveals no conclusion concerning why aborts seem to be more prevalent after the polymerase has spent time in the reactor. We can assume that the polymerase is not as stable when removed from the reactor, possibly due to the shearing effects, temperature, or a substance that denatures the polymerase. The higher amount of aborts suggest that the polymerase remains in the initiation state and is unable to catalyze elongation. This leads to a possible conclusion that the polymerase's tertiary and quaternary structure could be affected and thus the conformational change needed for chain elongation is unable to occur.

3.3.3 Membrane Compatibility

A membrane compatibility experiment was undertaken to assure that the membrane was not the reason for the decrease in transcription in the stir-cell. Figure 3.9 shows the electrophoresis gel from the membrane study, which indicates that the membrane does not affect the reaction. The image to the left shows a reaction in the stir-cell with a piece of Teflon in place of the membrane. What is seen is that very little product is made compared to the reaction with the membrane present. The reason for the stir-cell not producing RNA in this experiment was traced to a detergent which was used to clean the radioactivity from the reactor. This IsoClean detergent

Figure 3.9: Membrane compatibility experiment showing that RNA is produced when a membrane is present (right). The image to the left is the production of RNA in the stir-cell with a piece of Teflon instead of a membrane.

did not affect any other experiment in this chapter. Although no data can be concluded when the Teflon membrane is substituted for the real membrane, the fact

that transcription occurs with a membrane concludes that the membrane does not interfere with the reaction.

3.3.4 Agarose Bead Loss

A bead loss study was conducted to determine the amount of beads lost between reactions. The concentration of beads at the beginning of any reaction is about 14.72 +/- 4.00 beads per 0.0001 mL, whereas the amount of beads after a transcription reaction is done with timepoints being taken is 4.16 +/- 2.00 beads per .0001 mL. There are 3 times more beads at the beginning of the reaction as opposed to after a 3 hour transcription reaction. A material balance concludes that the system won't close. The loss of beads can be accounted for by beads being lost when retentate samples are taken, beads being left in the stir-cell when the reaction is over, and finally beads staying behind on the membrane. One difficulty is the use of a 0.1 mm deep slide for the hemacytometer which is designed for counting cells not agarose beads. The bead size distribution causes some of the beads not to diffuse under the microscope slide (0.1 mm). A better method is needed to count beads and determine the distribution.

3.3.5 RNA Degradation and Bead Stability

The purpose of using a stir-cell bioreactor is to potentially scale-up the process and use the beads and polymerase over a long period. To test the effects of stirring on the beads and the template, beads were allowed to remain in the stir-cell bioreactor for

12 and 24 hours in buffer with stirring. At these time points, transcription reagents were added to the reactor to determine if transcription can still occur. Figure 3.10 reveals that at 24 hours the beads still transcribe RNA. These data indicate that since RNA is still produced, the beads are very durable and the shearing force of the stir bar has no significant effect on the DNA template and its attachment to the agarose beads.

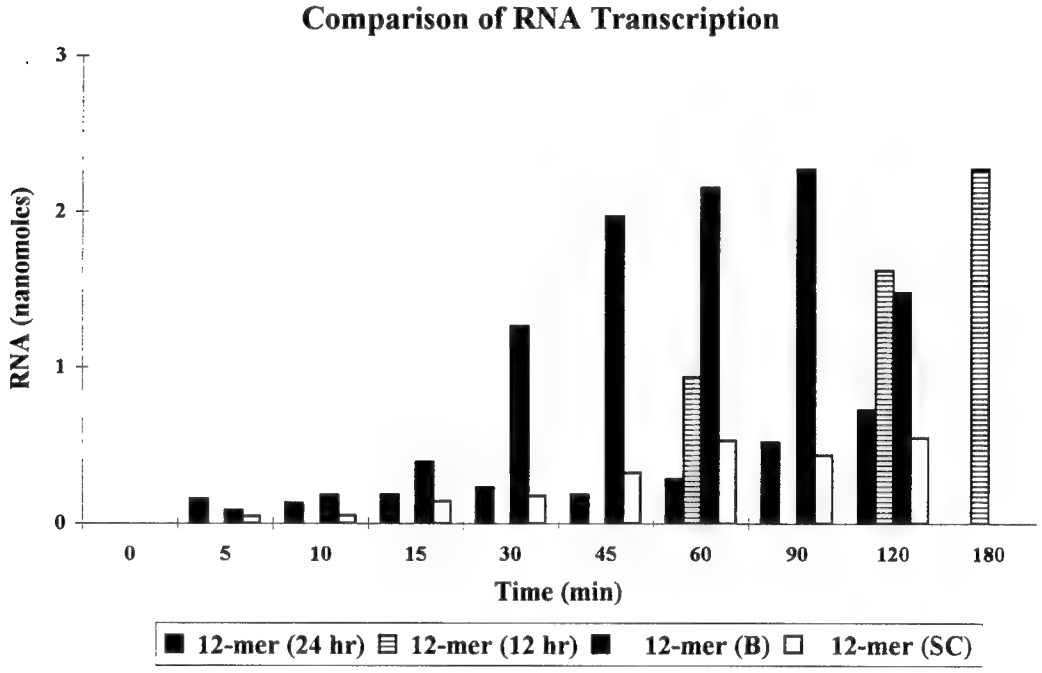


Figure 3.10: Transcription of RNA after the beads have been located in the bioreactor with stirring for 24 and 12 hours. For comparison the amounts of 12-mer produced in batch (B) and stir-cell (SC) with fresh beads are shown. Seventy percent of activity lost when the beads remain in the reactor for 24 hours.

A possible conclusion is the heat generated by the magnetic stir plate could be causing some denaturing of the DNA template. Beads are normally kept at 4 °C between transcription reactions

Additionally, if RNA is going to be made continuously over time, there is the possibility of the RNA being degraded while in the reactor. Labeled RNA products

including aborts from a transcription reaction were introduced to a reaction which had been reacting for up to 4 hours. Minor amounts of RNA were degraded within the first 3 hours, but degradation was definitely seen after 26 hours (Figure 3.11). The reaction of DNA to RNA is described below:

$$\text{NTPs} \xrightarrow[\text{DNA T7 Pol.}]{} \text{RNA} + \text{PP}i$$

Because of this reaction scheme, if pyrophosphate builds up over time, then the reaction could be driven the other way in which T7 polymerase begins to break RNA into NTPs. From the results in Figure 3.11, the data indicate that within the first 8 hours of a reaction the enzyme is not going backwards to degrade RNA because of the driving force of the increased amounts of pyrophosphate in the reaction. At 26 hours, it appears that the RNA is degrading, since the 12-mer is no longer seen and short

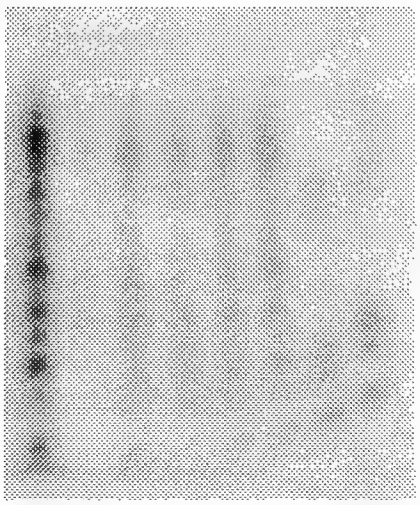


Figure 3.11: RNA degradation experiment showing that the within the first 4 hours the RNA is not degraded while after 26 hours significant degradation occurs. The S0 refers to the composition of the original material, R0 is the zero time point, and the following numbers indicate the hours after the mixture was added to the stir-cell.

aborts can be seen although whether the polymerase is catalyzing this reverse reaction is unknown. In a continuous process, RNA degradation would seem unlikely since the pyrophosphate would be continuously drawn out of the bioreactor through the membrane. The reason for this experiment was to determine if RNA was degraded in the stir-cell. If significant degradation occurs soon after a reaction is begun then the total amount of specific RNA made would be less because after being produced in the stir-cell it would then start to be degraded. Fortunately this study indicates that within four hours very little degradation occurs and only after 26 hours does this degradation become a problem.

3.4 Conclusions

The purpose of these studies was to examine the reaction materials to determine how these materials would react in the bioreactor with a membrane and the stirring action. The reason for these studies was to determine if any of these materials reacted differently in a bioreactor as opposed to small scale batch reactions. The beads adsorbed NTPs and RNA. This adsorption does not affect the transcription of RNA although for scale-up this adsorption might increase the cost. An alternative might be to use a different immobilizing matrix for the DNA template. The beads are durable and indicate that transcription occurs if the beads remain in the stir-cell for extended periods of time although a 70% loss of template activity occurs. Furthermore, RNA is not degraded unless the RNA is allowed to remain in the stir-cell for extended periods of time, which is unlikely in a continuous flow system. In

addition, the membrane shows no interference in the transcription of RNA. The potential drawbacks seen with set-up is that the beads are lost during the reaction and difficult to remove from the reactor. This bead loss is attributed through timepoint taking as well as the removal of the reaction materials after a reaction. Finally, the T7 polymerase appears to be denatured in the stir-cell reaction with time, indicating that the polymerase is no longer active and is unable to transcribe the full length RNA transcript.

CHAPTER IV

SEMI-CONTINUOUS RNA PRODUCTION IN A STIR-CELL BIOREACTOR

4.1 Objective

The objective of the semi-continuous stir-cell RNA reactions was to determine the feasibility of using a bioreactor for continuous production of RNA. Further studies are needed to fully understand the transcription reaction before further scale-up is attempted. After characterizing the effects of the reaction materials in the stir-cell (Chapter III), RNA transcription was performed semi-continuously in the stir-cell bioreactor without stirring to determine the effects of the impeller as well as transcription using solution phase DNA template (without beads) before other transcription studies were performed. These studies were done to determine the effects of transcription in combination with the results from Chapter III and to see if these effects actually interfered on the production scale. Once RNA transcription was proven to occur within the stir-cell, the rate of RNA production over time versus a batch reaction was determined. Furthermore, ten-hour reactions were accomplished to simulate continuous RNA production in both batch and the stir-cell. In addition,

optimized NTP concentrations were used to show that more RNA could be produced if the initial reaction conditions were changed. Finally, an enhanced method of RNA production was used from the observations of the previous studies to show that an optimized flow of reactants into the reaction could boost the production of RNA.

4.2 Materials and Methods

4.2.1 RNA Transcription Reaction

The basic transcription reaction used for all these studies is similar to the reaction described in Chapters II and III. This transcription reaction was used to characterize production of RNA within the stir-cell and batch reactions. The materials listed below were used in both radioactive and non-radioactive reactions with radioactive studies incorporating radioactively-labeled uridine triphosphate. The materials in the reactions were a standard transcription reaction mixture containing:

40 mM Tris-HCl (pH 8.1)	1.0 mM Adenosine Triphosphate
20 mM Magnesium Chloride	1.0 mM Cytosine Triphosphate
5 mM Dithiothreitol	1.0 mM Guanosine Triphosphate
1 mM Spermidine-HCl	0.1 mM Uridine Triphosphate
0.01 % Triton X-100	0.125 μM [α-^{32}P]-UTP (10 μCi)
1 μM DNA Template on beads	0.023 mg/mL T7 RNA Polymerase

This mixture was introduced to the stir-cell and/or batch. The above components were used and described as the standardized transcription mixture. The cold UTP concentration is 0.1 mM to improve the signal from the radioactive UTP. In contrast, stoichiometric amounts of NTPs were used as the optimal amounts of NTPs: 8.004 mM G, 4.819 mM U, and 6.405 mM C.

4.2.2 Transcription Reaction with No Mixing

A transcription-reaction experiment was performed in the stir-cell without stirring or mixing for comparison to a batch reaction with mixing. The reason for such an experiment is to test transcription in the stir-cell without mixing to determine if the stir-cell and the stir-bar affect the production of RNA. At each hour, 600 μL were removed from the retentate within the stir cell and batch, with an addition of NTPs, buffer, and d_AH_2O back into the reactions to keep a constant volume. These samples were loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was scanned on the PhosphorImager.

4.2.3 Solution Phase Transcription in the Stir-cell

In previous studies (Chapter III), data had shown that the beads adsorbed NTPs to a considerable degree. If these beads then sediment the adsorbed NTPs are no longer in suspension and thus are unusable to the reaction until they desorb. If this is one reason for the decrease of transcription then a solution phase reaction should eliminate this loss of NTPs. From a solution of 10 μM of a top and bottom strand of 28-mer, 45 μL of each was added and placed in a heating unit (Thermolyne Type 16500 Dri Heat) at 90 degrees Celsius for 5 minutes then put in ice for 30 minutes to anneal. A 900 μL transcription reaction was set up in the stir cell without beads and 100 μL of **retentate** was removed at 0, 30, 60, 90, 120, and 180 minutes. NTPs,

buffer, and d_AH_2O were added back to keep the volume constant. Although with each timepoint, the overall concentration of DNA template decreases there should be enough template to determine if transcription is occurring in the stir-cell. No samples were removed through the membrane since the template is not immobilized. These samples were loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was scanned on the PhosphorImager.

4.2.4 Rate of RNA Transcription: Batch versus Stir-cell

A rate study was also performed using a 1000 μL transcription reaction in batch and the stir-cell bioreactor. From the batch reaction, 20 μL samples were taken, as were 20 μL samples from the retentate side of the stir-cell. No permeate samples were taken and no solution was added back to the reactions. Timepoints were taken at 0, 5, 10, 15, 30, 45, 60, 90, and 120 minutes. To these samples 5 μL of 75% formamide was added, and then they were loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager.

4.2.5 RNA Transcription over Time: Batch versus Stir-cell

A study was undertaken to chart RNA production over a 10 hour period in both batch and stir-cell to determine the course of the transcription reaction and to

determine if adding polymerase late in the reaction allows for a boost in transcription. Two 1000 μL transcription reactions were set up, and every hour 500 μL of permeate sample was withdrawn through the membrane using the syringe, and, a 20 μL retentate sample was also taken. A 520 μL sample was removed from the batch reaction and, to both reactions, 520 μL of buffer, NTPs, and d_AH_2O was added back. After the 8 hour timepoint was taken, 20 μL of T7 polymerase was added to both reactions. From the 520 μL samples, 20 μL was removed and added to 5 μL of 75% formamide and loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager. This experiment was repeated following the above procedure except fresh NTPs were used as well as a new bead preparation.

4.2.6 RNA Production Characteristics over Time: Batch versus Stir-cell

A series of transcription reactions was accomplished in batch and the stir-cell to determine if the RNA transcription was continuous and to determine the time when NTPs were depleted. Questions have arisen about whether the transcription reaction in the bioreactor is continuous over a long period of time. Labeled UTP was added at the beginning of the reaction, and samples were continuously removed at each timepoint. It is assumed that transcripts are continuously produced. Unfortunately, over a two-hour reaction, RNA could be made within the first 10 minutes and then this RNA might be continuously withdrawn over a two-hour period. To test this, a cold

(non-radioactive) RNA transcription reaction was set up in batch and in the stir-cell and allowed to run for a specified amount of time. When this time was reached, labeled UTP was inserted into the reaction and an hour later a timepoint was taken. Through a series of experiments, the reaction was allowed to run for a specific amount of time before adding labeled UTP and thus a profile of RNA transcription of specific and product RNA (aborts) can be determined. Labeled UTP was added at 0, 1, 2, 3, and 4 hours with a profile of RNA production showing the amount produced from the interval of 0 to 1 hour, 1 to 2 hours, 2 to 3 hours, 3 to 4 hours, and 4 to 5 hours.

4.2.7 Optimizing NTPs versus Standard Reaction Conditions

The traditional transcription reaction described in section 4.2.1 used equimolar NTP concentrations except for limiting UTP (1 mM A, G, C and .1 mM U). This mix was used because it was easy to make and allowed for more of the labeled UTP to be incorporated into the transcription products which gave an adequate signal to be viewed with the PhosphorImager. Unfortunately, these concentrations are not the optimal ones. Optimal conditions need to be designed with specific knowledge of the sequence which is to be produced in transcription. The dodecamer contains 5 G's, 4 C's, 3 U's, and no A's. Notice that the traditional reaction contains A, which is not needed in the 12-mer being studied. Knowing the sequence, a stoichiometric amount of NTPs was used to compare transcription with the traditional mix of NTPs. The new concentration of NTPs contained 8.004 mM G, 4.819 mM U and 6.405 mM C.

This particular choice has been called optimal and was picked from a series of experiments run in the lab (33).

An initial experiment was performed to determine the effects of the optimal NTP concentrations in batch and stir-cell and compare these results to a reaction with standard NTP concentrations in a batch and stir-cell. Four 1000 μL transcription reactions were set up with the conditions described in section 4.2.1. At the timepoints of 1, 3 and 4 hours, 20 μL of sample was removed from batch and from the retentate side of the membrane. No solution was added back to the reactor, and to each sample 5 μL of 75% formamide were added and then the sample was loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager.

In addition to the experiment described in section 4.2.6, a reaction was performed as described in this section except the optimal NTP concentrations were used and labeled UTP was added to the reaction at different times, giving a profile of RNA production over time. The reason for this experiment was to determine how the RNA production profile changed when different NTP concentrations were used. Additionally, the results would help to determine the effects seen from data found in section 4.2.6.

Two 100 μL transcription reactions were set up in batch. The reason for doing a batch reaction was to perform a lane by lane comparison of using optimal and standard conditions. The reactions contained the mixture described in section 4.2.1.

An initial timepoint was taken with all the components except T7 polymerase. The polymerase was then added and the reaction begun. Samples were removed at 5, 10, 15, 30, 45, and 60 minutes. These samples were added to 5 μL of 75% formamide and loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager.

The final optimized reactions were done to determine the rate of RNA transcription in batch and stir-cell using optimized conditions. Two 1000 μL transcription reactions were set up using the conditions described in section 4.2.1 except optimized NTP concentrations were used. At 5, 10, 15, 30, 45, 60, 90, 120, 180 minutes, 20 μL of retentate from the stir-cell was removed and the same were removed from the batch reactor. From the 20 μL, 10 μL was removed and added to 5 μL of 75% formamide and loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager. The remaining 10 μL was stored in the freezer.

4.2.8 Simulation of Continuous RNA Production

A 1000 μL transcription reaction was set up in the bioreactor. Limiting UTP conditions were used for this experiment. 100 μL retentate samples were taken at 15, 30, 60, and 90 minutes. After the 90 minute timepoint was taken, a sample containing 40 μL NTPs, 40 μL T7 buffer, 20 μL T7 polymerase, 1 μL UTP^{32}, and 300 μL d_AH_2O

was added back to the reactor. After addition at the 90 minute timepoint, 100 μL samples were taken at 105, 120, 150, and 180 minutes. Once again after the 180 minute timepoint a replacement solution of NTPs, buffer, and polymerase was added containing the same mixture as in the 90-minute timepoint. This procedure was repeated with timepoints taken at 195, 210, 240, and 270 minutes with an addition at 270 minutes and continued timepoints at 285, 300, and 330 minutes. From these samples, 20 μL was removed and added to 5 μL of 75% formamide and this sample was loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager.

4.3 Results and Discussion

4.3.1 Transcription Reaction with No Mixing

With no mixing in the stir-cell, the transcription reaction did not produce any significant amounts of RNA. This is to be expected, for the transcription reaction to occur it would need to be well mixed, otherwise the components would not have enough contact to react with one another. No mixing would also assure that the beads had sedimented to the surface of the membrane. This allows one possible explanation of decreased transcription in the stir-cell. If the beads sediment to the surface or a fraction of these beads stick to the membrane, then the DNA template which is carried

along with the beads sediments. The sedimentation of beads, if the average diameter is 50 microns and assume density of about 1 g/cm^3, is 6 mm/min while the size of the polymerase is 70 Å which would indicate a sedimentation rate of .0007 mm/min (14).

The polymerase has a high affinity for the promoter region of the DNA template, so if the template is not in solution then the polymerase would have less chance of being used in the formation of new RNA. In a typical reaction, there exists 1 μM DNA to .257 μM T7 polymerase. There would be four DNA templates for every molecule of polymerase. Thus, if polymerase does have affinity for DNA then the polymerase would be attached to the DNA templates which have sedimented to the membrane surface and very little polymerase would remain in solution. Since the system is not mixed the reactants do not reach the polymerase and template as effectively, and thus the system does not produce any significant amounts of RNA. With this procedure one would expect the polymerase to be removed with time, but the total fraction of polymerase is not significant.

4.3.2 Solution Phase Transcription in the Stir-cell

The beads have been shown to adsorb NTPs and RNA (Chapters II and III) and, because of this adsorption the beads could be affecting the reaction by removing reactants from the solution and trapping the NTPs in the pores of the beads. To determine if this is occurring, a solution phase reaction was done using solution phase DNA template. Results indicate that hardly any RNA is produced (Figure 4.1). Reasons why no transcription is occurring is that the concentration of DNA might

have been less than expected or possibly the template did not fully anneal. Again the procedure allows for the removal of template with time but the amount of template removed is insignificant especially since no transcription is seen to occur. One interesting note is that with a solution phase reaction the membrane adsorbs more of the labeled UTP in previous immobilization transcription reactions, thus revealing that the majority of labeled UTP is adsorbed by the agarose beads when they are present. This result indicates another importance of beads. The NTPs would stay near the reaction in the beads rather then being adsorbed by the membrane. This is especially important when a membrane is used to separate the components of the reaction because, if the majority of the NTPs are

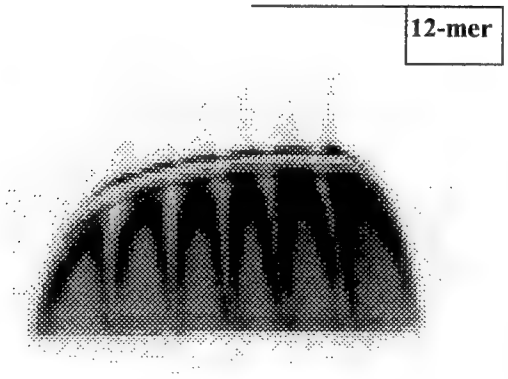


Figure 4.1: Electrophoretic gel reveals that very little RNA is produced with solution phase templates in the stir-cell.

adsorbed by the beads, then these NTPs would be retained in the reaction vessel. In a continuous process the beads would be integral in retaining the NTPs longer in the reactor and prevent the quick exit of these lower molecular weight monomers.

4.3.3 Rate of RNA Transcription: Batch versus Stir-cell

A series of comparison studies was done to compare RNA transcription in batch and the stir-cell bioreactor. Figure 4.2 shows that the rate of transcription in the batch reaction is higher than that in the stir-cell. The product lines on Figure 4.2 (total RNA) signify the 12-mer and all the smaller aborts, while the 12-mer is just the band that is predicted to be the RNA that is being studied. The graph below supports, among others, the idea that using limiting UTP conditions the reaction rate reaches a maximum because the NTPs are being used up very quickly, and RNA production plateaus because no building blocks are present for the polymerase to produce RNA.

The amount of RNA was determined with the help of the ImageQuant software on the PhosphorImager. The bands on the gel image were first circled as was the entire lane containing the RNA bands and the unincorporated UTP. The integrated intensity of the product band was divided by that of the entire lane ,and this ratio was considered the percentage of labeled UTP incorporated into the RNA transcript. This ratio was then divided by three which is equal to the number of U's in the RNA sequence and then this value is multiplied by the concentration of UTP in the reaction and then multiplied by the volume of reaction. This value is the number of moles of RNA produced. This procedure was used for all the results presented in this chapter and additional chapters. Table 4.1 describes the rate of incorporation from Figure 4.2.

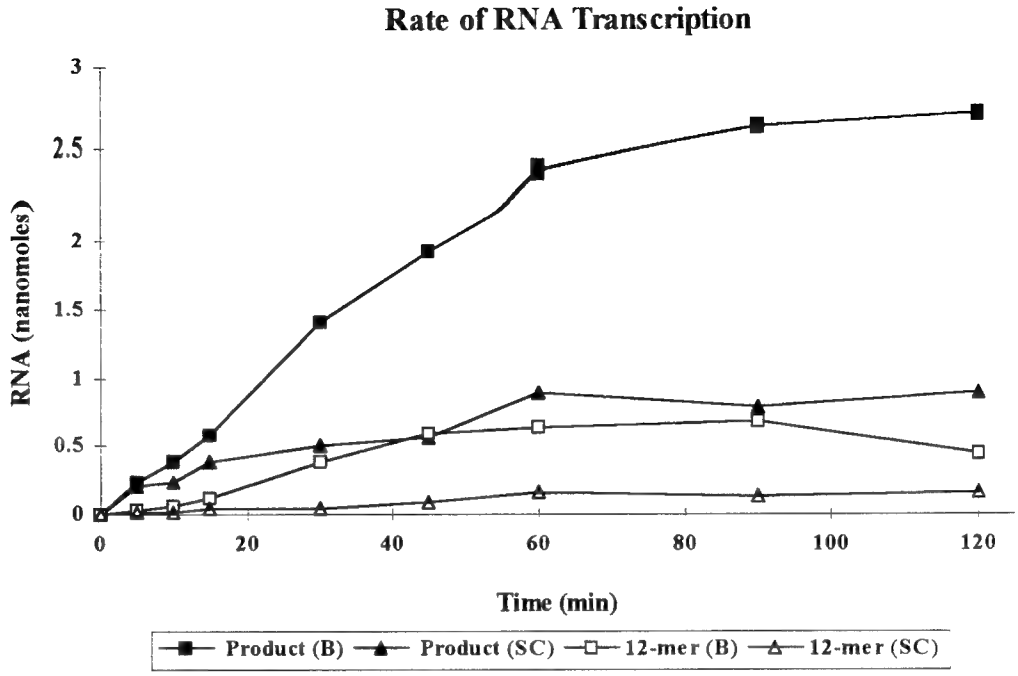


Figure 4.2: RNA transcription versus time in the stir-cell (SC) and batch (B)

Table 4.1
Comparison of the Incorporation Rates for Batch and the Stir-cell comparing Product and 12-mer Rates

	Reaction	Rate of Incorporation (nanomoles/mL*min)
Stir-cell	Product	0.0150
	12-mer	0.0024
Batch	Product	0.0417
	12-mer	0.0115

4.3.4 RNA Transcription over Time: Batch versus Stir-cell

The initial rates of RNA transcription are important, but to create conditions that would approach a continuous process, longer reaction times need to be

considered. A series of 10-hour reactions was done to chart the production characteristics of RNA in the stir-cell and batch. After each hour, half the volume was removed and replaced with fresh buffer and NTPs. At the 8 hour timepoint fresh polymerase was also added to the reactions. From Figure 4.3 it can be seen that transcription of the 12-mer continues to increase, levels off, then steadily decreases until fresh polymerase was added and then the amount of RNA increases. Percent incorporation was the amount of labeled UTP that was incorporated into specific RNA (12-mer). The leveling off seen was interesting in that the polymerase should not be running out of reaction materials like NTPs. Another interesting point was that polymerase was steadily removed or diluted with time in the batch reactions. Meanwhile, in the stir-cell, half of the volume was removed through the membrane but the polymerase should be retained. Figure 4.4 shows the cumulative production of RNA; the stir-cell does outproduce the batch reaction but the difference was very small. One should expect a greater difference since more polymerase should remain in the stir-cell, thus allowing for more production of RNA. Reasons for this include the possibility of polymerase associating with the templates and thus not being removed with the samples from the batch reactor. Another possibility was that the polymerase is deactivating with time, as demonstrated in section 3.3.2, which would explain the decrease in the overall RNA production which is seen in Figure 4.3. Furthermore, what can be seen is that, by adding fresh polymerase, the production of RNA can be brought back up to previous levels. Reasons for the large error bars seen in Figure 4.3 are due to changing reaction conditions. The concentrations were not changed in the

reactions, but new NTP solutions were made up and fresh beads were used. What can be inferred is that the reaction is very sensitive to the freshness of materials. This can be seen in Figures 4.5 and 4.6 which show the importance of fresh materials. The used beads had been recycled from previous reactions and a conclusion is that over time the beads' DNA is lost. The freshness of a NTP solution is important as well. NTPs break down over time when put in solution so a solution of NTPs that have been frozen over a couple of months will be less effective than a newly formed batch of NTPs.

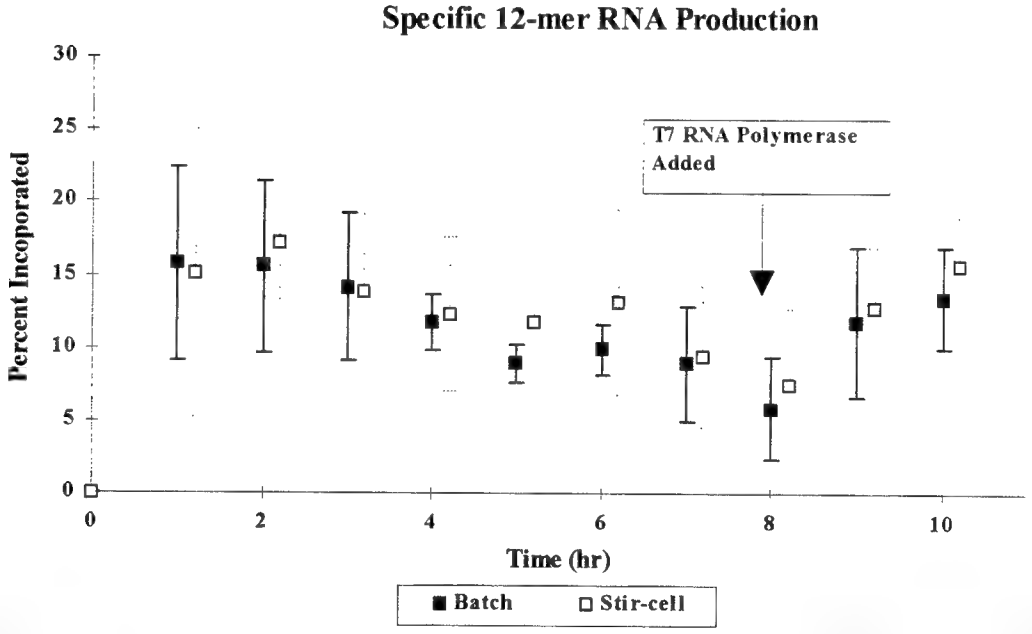


Figure 4.3: RNA production in the stir-cell and batch showing that RNA production declines and then can be revitalized with the addition of fresh polymerase. The error bars represent plus and minus one standard deviation about the mean for three repetitions for both batch and reactor.

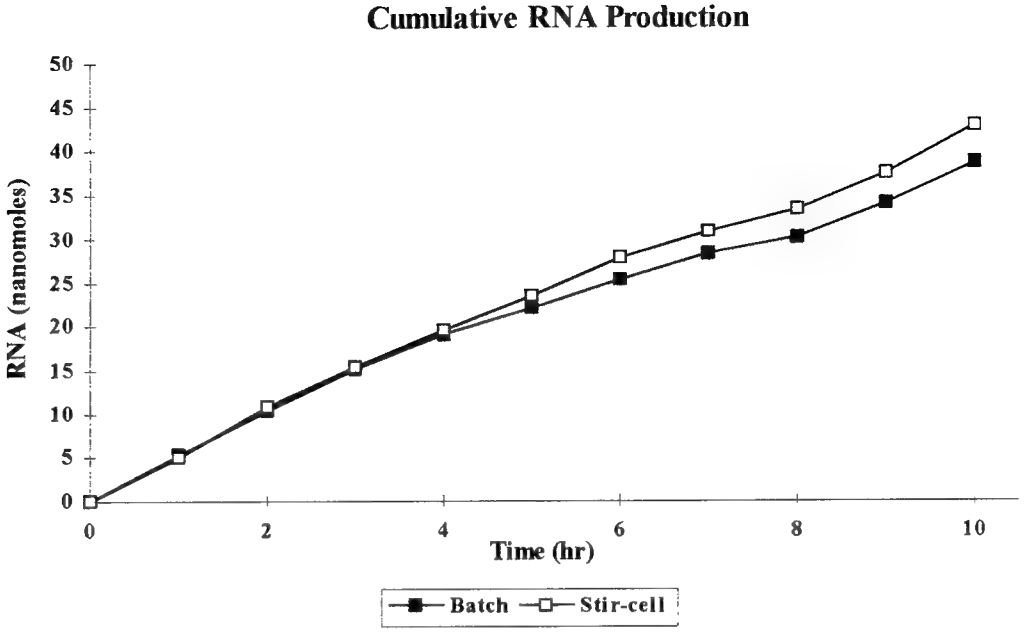


Figure 4.4: Cumulative RNA production showing that the retention of polymerase in the stir-cell does not drastically change the overall amount of total RNA produced.

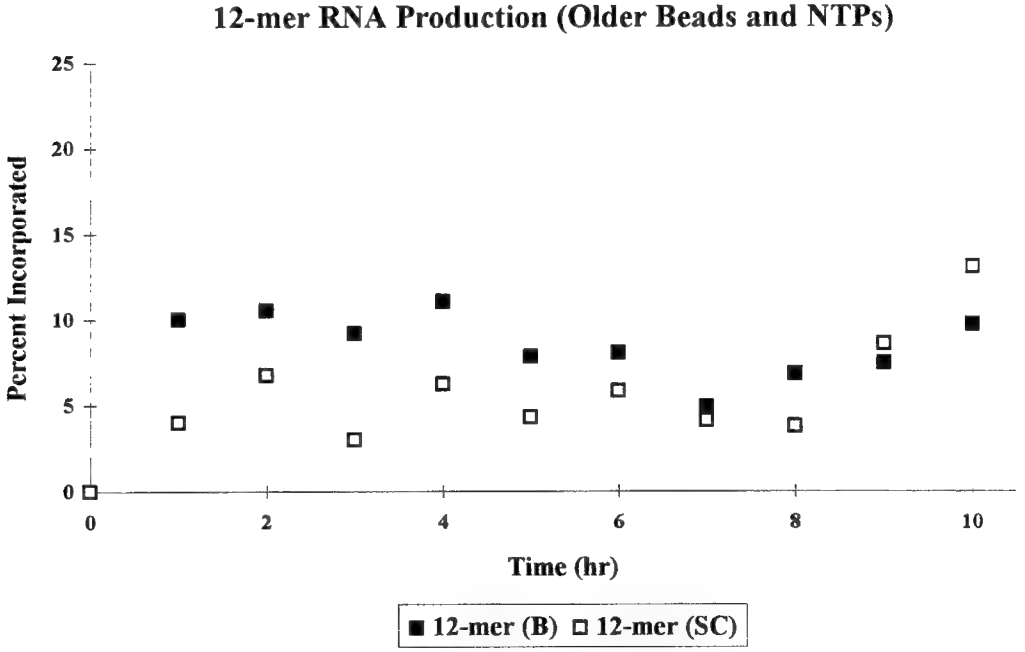


Figure 4.5: Specific RNA production in batch (B) and the stir-cell (SC) using used beads and an older batch of NTP solution. Compare this with figure 4.6 which used fresh beads and a new NTP solution.

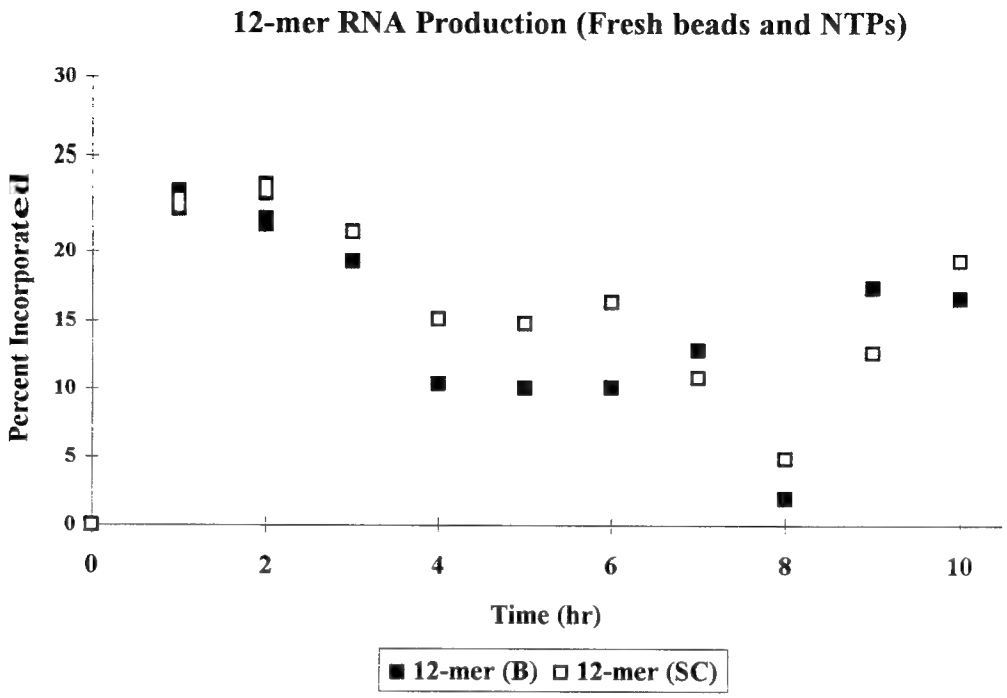


Figure 4.6: Specific RNA production in batch (B) and the stir-cell (SC) showing that depending on the freshness of the materials used the amount of product made is increased.

4.3.5 RNA Production Characteristics over Time: Batch versus Stir-cell

The reactions that were done previously using radioactive labeled material (UTP) cannot answer the question of whether RNA is continuously being produced and what the transcription reaction is doing at later timepoints. This problem occurs because the labeled reactants are added at the zero timepoint, and at later timepoints when the product is being removed there is no way of determining when it was formed. This is because the labeled material could have been made within the first hour of transcription and the later timepoints could be just diluting this previously made material. To determine if transcription continues over a long period of time, labeled UTP was added at specific times and allowed to react for only a specific time

period. This allows us to track the transcription process over time. The results can be seen in Figure 4.7. The graph indicates that specific 12-mer RNA is made within the first hour with some minor amount being made in the second hour. The amount of total RNA, which includes aborts and desired product, peaks well within the first two hours, levels off, then begins to increase, but without a corresponding increase in desired product (12-mer) RNA production. This reaction was run with the standard reaction conditions with limiting cold UTP concentrations (1 mM A,T,C and .1 mM U).

The increase in aborts at later timepoints can be explained with three possible reasons. The first is that the reaction is running out of GTP. These reactions are not adding back NTPs and buffer since the reactions are allowed to run then at specific times after labeled UTP is added. Since G starts the 12-mer sequence, the aborts and specific product are rich in G and it is possible that polymerase will start mis-incorporating other NTPs that are present if G is not present. A second explanation is that, at the later timepoints (3-5 hours), a high concentration of abortive sequences is present when fresh labeled UTP is added. The polymerase uses these aborts to initiate transcription and adds the labeled UTP, but does not have enough of the other NTPs to continue transcription. The fresh labeled UTP could also be added to previous aborts that the polymerase initiates with and then adds the UTP. The final possible reason is that degradation is occurring. Once again, at the later timepoints non-labeled RNA exists, and if a high concentration of pyrophosphate exists then the polymerase

could be going backwards and reducing RNA to NTPs. The T7 polymerase is an enzyme capable of being reversible:

$$\text{NTPs} \xrightarrow[\text{DNA + T7 Pol.}]{} \text{RNA} + PP_i$$

Another trend seen in Figure 4.7 is the onset of these events seems to occur later in the stir-cell than in batch. This means that the rate of transcription in the stir-cell is lower because the stir-cell might affect the reaction materials. This is a potential drawback, if time is important in scale-up, as the stir-cell will take longer to produce RNA.

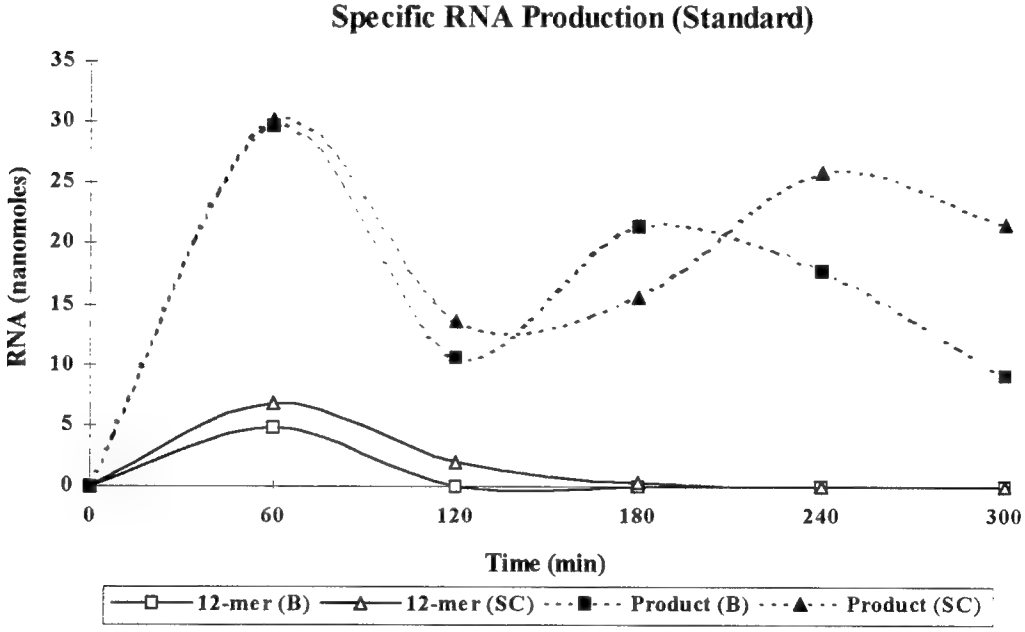


Figure 4.7: Specific RNA production showing the product distribution at each timepoint indicating that RNA is continuously produced,

The above results indicate that the NTPs are being used up very quickly in the reaction because the amount of desired RNA produced after 120 minutes is minimal. In addition, the amount of aborts generated is also decreasing from 60 to 120 minutes

so if NTPs were present this decrease in aborts and desired product would still be increasing or reaching a plateau instead of dropping off. To determine why the results from Figure 4.7 occur, an optimal amount of NTPs was used to determine how the specific RNA production is affected. This optimal amount of NTPs consisted of a stoichiometric amount of NTPs: 8.0 mM GTP, 6.4 mM CTP, and 4.0 mM UTP. Notice this solution of NTPs has no ATP since this nucleotide is not needed for the production of the 12-mer. The results of using an optimal amount of NTPs can be seen in Figure 4.8.

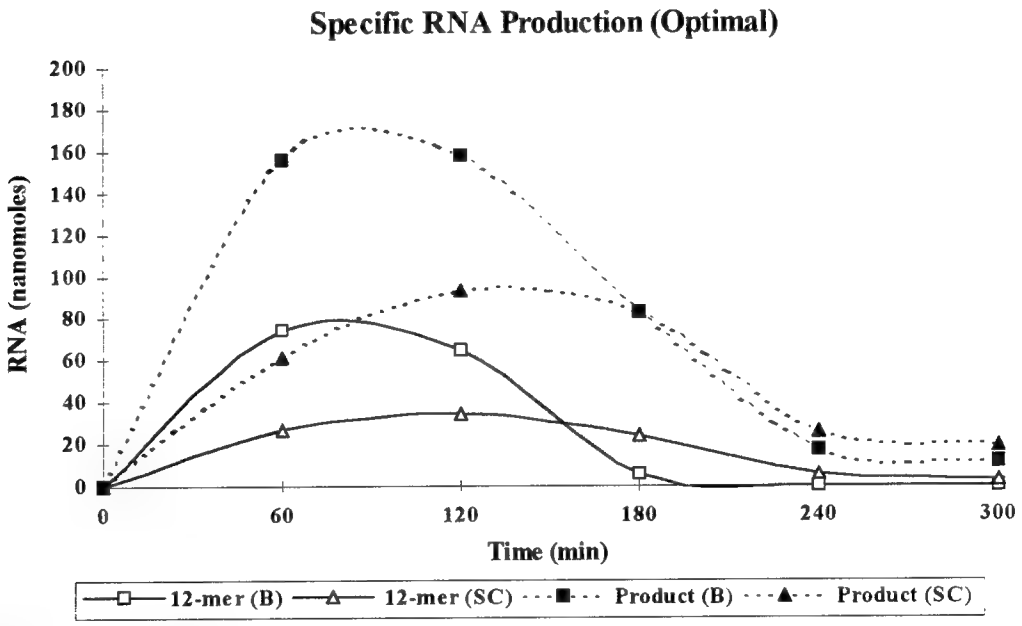


Figure 4.8: Specific RNA production using optimal NTP concentrations at time intervals of one hour.

By using a more optimal amount of NTPs one can extend the production of RNA to about 90 to 120 minutes from the 60 to 90 minutes using the standard NTP conditions. Another result is that, within the 5 hours that the reaction was run, the increase of aborts is not seen, which means that the results seen in Figure 4.7 could be

due to because polymerase mis-incorporating NTPs, probably ATP. Also, the results in Chapter III show that very little RNA degradation occurs in the stir-cell from the period of 5 to 8 hours. Also, the amount of RNA produced is greater using the optimal amounts of RNA. A peak of 35 nanomoles is made with optimal conditions in the stir-cell, while only 5 nanomoles is made for limiting conditions. One nanomoles of RNA (12-mer) is equal to about 4 μg of RNA.

4.3.6 Optimizing NTPs versus Standard Reaction Conditions

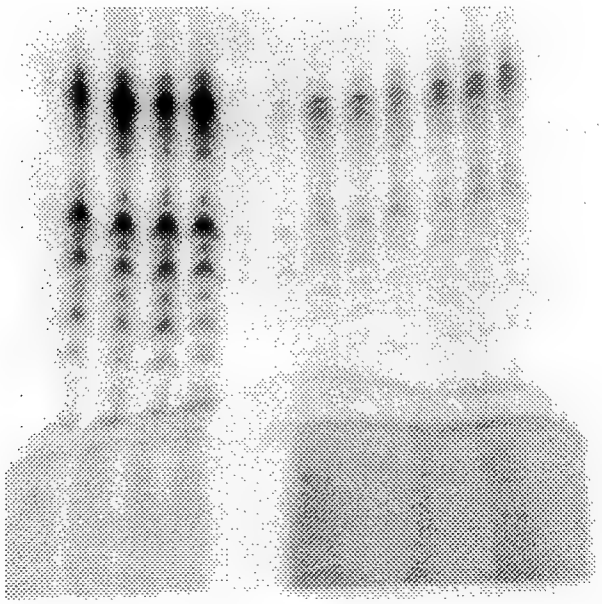
From the above studies, using optimized NTP conditions increases the amount of RNA that can be made. An initial study, which can be seen in Figure 4.9 is a comparison of transcription in batch and the stir-cell with standard and optimizing conditions. The picture shows that with a lower cold UTP concentration (standard, 0.1 mM UTP) the signal is much better (darker bands) but with the optimized NTP concentrations the amount of total aborts is less. Additionally, in Figure 4.10 a lane-by-lane comparison can be seen using standard and optimizing NTP concentrations in batch.

From Chapter III, the percent of abortive termination at a certain position in the 12-mer was introduced to show quantitatively the amounts of aborts at specific bases in the RNA sequence. Figure 4.11 compares a typical RNA transcription reaction using the standard NTP conditions in which 0.1 mM UTP is present and the optimized NTP conditions in which 4.0 mM UTP is present. UTP becomes limiting in the standard conditions which results in more aborts at the first U in the RNA

sequence. Depletion of NTPs is more likely using equimolar NTP concentrations, because a certain sequence has different needs for different NTPs. A solution designed with a specific sequence in mind will yield more RNA. In the transcription of the dodecamer, the use of a greater concentration of GTP makes sense because this NTP is more prevalent in the sequence, especially in the abort sequences. In Figure 4.9 and 4.10, bands can be seen below the 6-mer. These bands are difficult to explain because the first U in the 12-mer sequence is position six:

GGC GCU UGC GUC

These bands can be attributed to misincorporation by the polymerase. In addition, the polymerase could be binding to the template or possibly RNA transcripts and transcribing without using the promoter to initiate binding.



B1 SC1 B0 SC0 B1 SC1 B3 SC3 B4 SC4

Figure 4.9: The picture indicates the differences between using standard and optimal conditions. Although the signal is better with standard conditions (left) the amount of aborts is less using the optimized conditions (right).

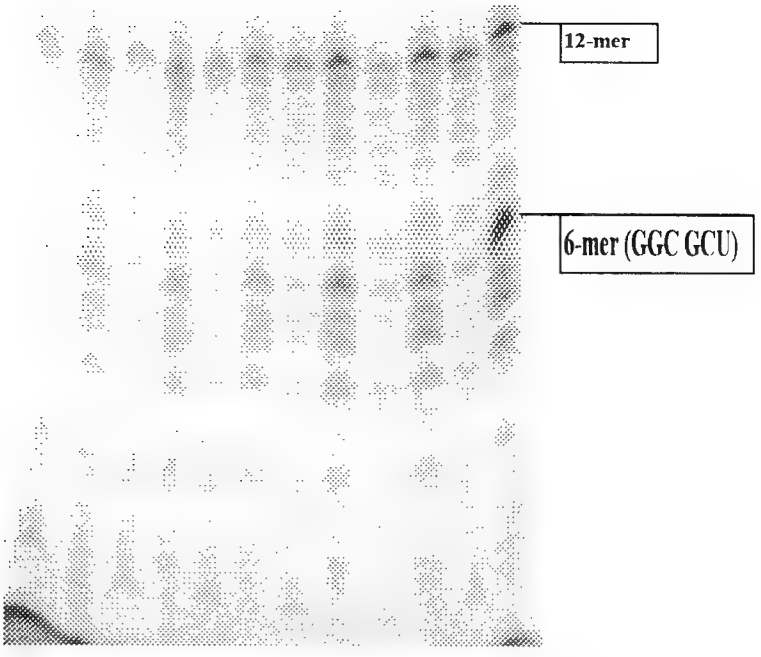


Figure 4.10: Electrophoretic picture of a transcription reaction comparing optimal (O) and standard (S) NTPs concentrations. The positions noted are the 12-mer and, counting down from this position, the 6-mer or the first transcript with a U. These positions are assumed to be the transcripts noted although no sequencing has been done to determine these positions. The numbers represent time in minutes.

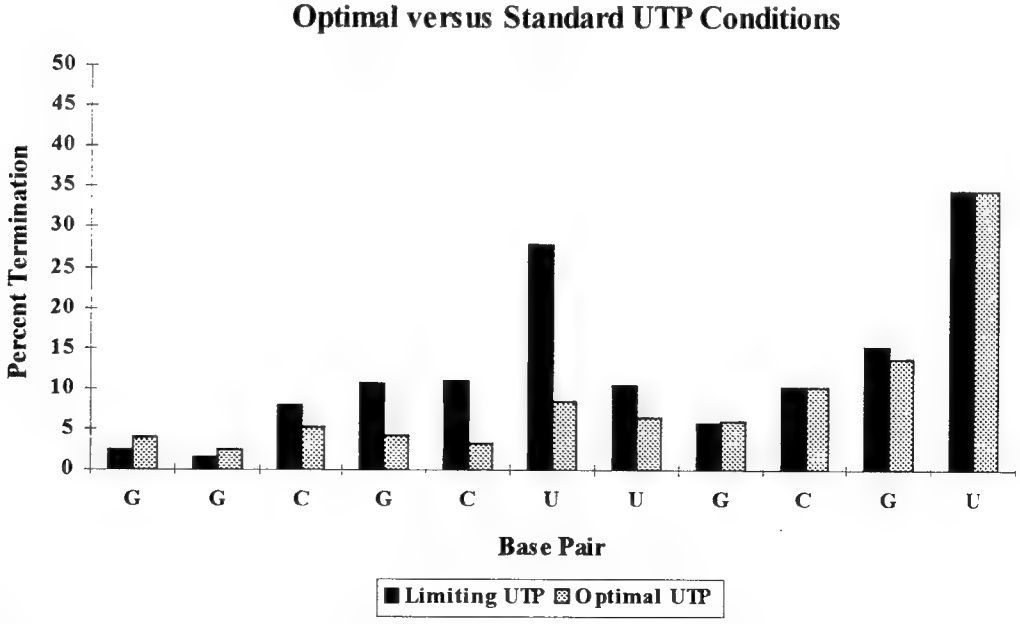


Figure 4.11: Percent termination of the developing RNA transcript showing that more aborts occur at the first U in the sequence when UTP is limiting (.1 mM compared to 4 mM).

The amount of RNA transcription versus time in both the stir-cell and batch bioreactor with both optimal and standard NTP concentrations are shown in Figure 4.12. In the stir-cell the initial rates are similar but the maximum rate is about two times faster under the standard NTP concentrations as opposed to optimized conditions. The batch reaction shows that the standard NTP conditions are two times faster initially but the maximum rates are similar. In Figures 4.13 and 4.14 the cumulative RNA production is shown for the various conditions. The amount produced in the same amount of time in the batch reaction is 40 times more in the optimal conditions than found in the standard conditions, while the stir-cell bioreactor

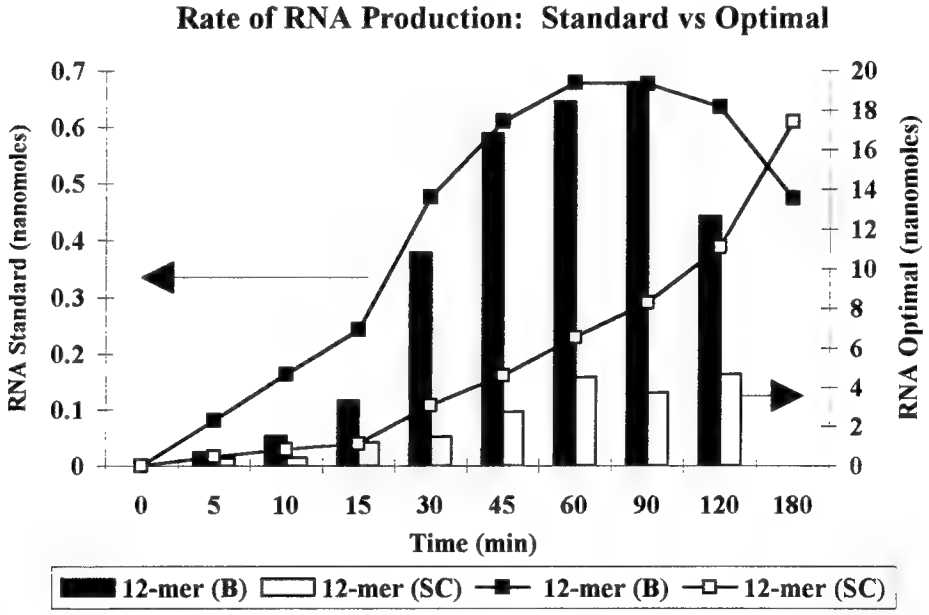


Figure 4.12: Comparison of rate of transcription of RNA comparing standard and optimized UTP concentrations. The standard is represented by the line graphs and the optimal reaction is represented by the bar graphs. The rate profiles appear to be similar but the amount of actual RNA produced is much greater in the optimal reactions.

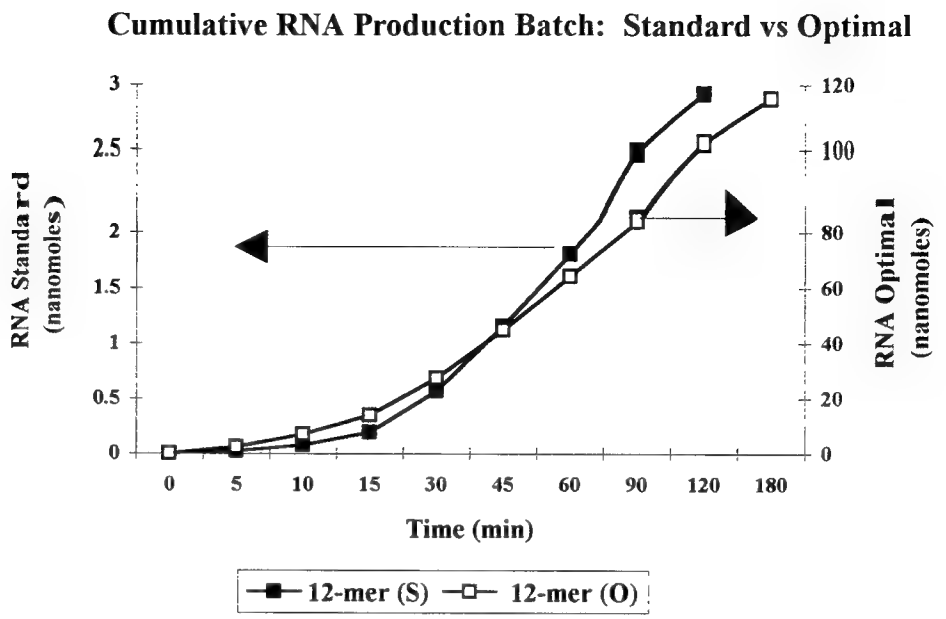


Figure 4.13: Cumulative RNA production in the batch reactor. Data taken from Figure 4.10 were summed to determine the total amount of RNA made over the 3 hour period rather than that produced at each time interval. The arrows point to the appropriate ordinate corresponding to the two conditions.

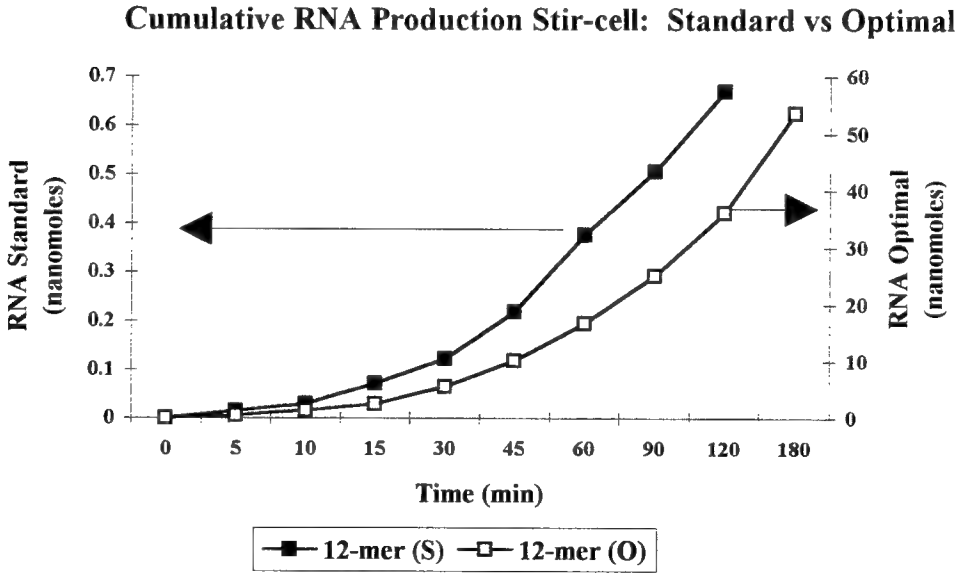


Figure 4.14 Cumulative RNA production in the stir-cell bioreactor. Data taken from Figure 4.10 were summed to determine the total amount of RNA made over the 3 hour period rather than that produced at each time interval. The arrows point to the appropriate ordinate corresponding to the two conditions.

produces 80 times more using the optimal NTP concentrations. These studies indicate that using optimal NTP concentrations is vital for producing more RNA and how NTPs affect the overall yield of producing RNA.

Table 4.2
Comparison of the Production Amounts (12-mer) for Batch and the Stir-cell using Standard and Optimized NTP Concentrations

	Reaction	Amount Produced (nanomoles)
Stir-cell	Standard	0.68
	Optimize	54
Batch	Standard	2.9
	Optimize	118

4.3.7 Simulation of Continuous RNA Production

Taking these results, a simulation of continuous RNA production can be undertaken. In Figures 4.2 and 4.3, the data indicate that in the range of 1 and 2 hours is when the production of RNA peaks, so instead of following the 10 hour reaction conditions where just buffer and NTPs are added every hour, this solution along with polymerase was added every 90 minutes. Figure 4.15 shows that RNA production can be increased over the ten hour cumulative production. This graph compares the 10 hour experiment described previously, which used 20 μL of polymerase initially and then an additional 20 μL at the 8 hour point, to give a production of about 40 nanomoles of desired RNA in 600 minutes. This is compared to the experiment in which 20 μL of polymerase is added initially and every 90 minutes 20 μL of additional polymerase is added. In a 300 minute time period, about 50 nanomoles of RNA was

produced. These experiments show that depending on the strategy, one can either spend more to make RNA faster or wait longer and use less money to produce the same amount of RNA.

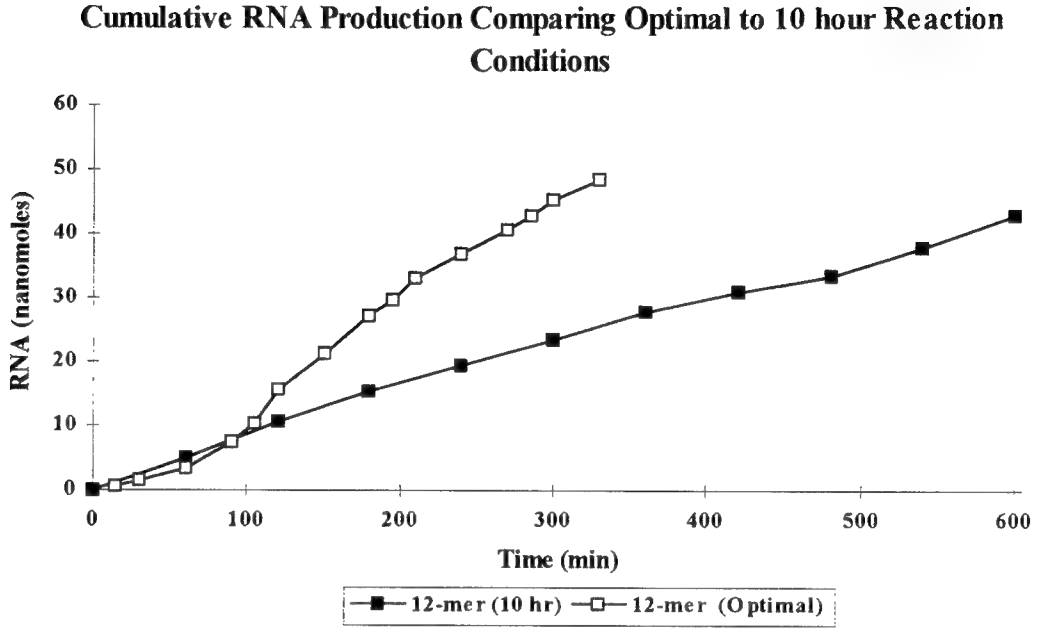


Figure 4.15: Simulation of continuous RNA productions shows that if polymerase is added continuously with buffer and NTPs then the amount of RNA produced can be enhanced although this enhancement is done with 80 μL of polymerase as opposed to 40 μL in the 10 hour experiments.

4.4 Conclusions

The results of the semi-continuous RNA production in the stir-cell suggest that RNA is capable of being produced in a continuous flow bioreactor. The rate of production of RNA is slower in the stir-cell but over a long period of transcription (10 hours) the cumulative amount of RNA produced is equivalent. Furthermore from these long transcription reactions it is evident that the reason for the decrease in RNA production over time is the inability of the polymerase to continue transcription. Reasons for this conclusion is that even with continued NTPs and buffer addition the

reaction continues to decrease in rate and only increases when fresh polymerase is added to the reaction. The importance of optimizing the NTP concentrations per RNA transcript is very important since an increase in yield will be obtained. Finally with an optimized addition of NTPs, buffer, and polymerase which simulates a continuous feed more RNA is produced indicating that a continuous bioreactor is feasible.

CHAPTER V

CONTINUOUS RNA PRODUCTION IN THE STIR-CELL MEMBRANE BIOREACTOR

5.1 Objective

The objective of the previous chapters was to determine the feasibility and usability of continuously making RNA in a stir-cell bioreactor using an ultrafiltration membrane to retain the DNA template and T7 polymerase and separate the newly formed RNA transcripts. The previous chapters have successfully shown that RNA can be separated using an ultrafilitration membrane, that RNA can be made in a stir-cell bioreactor, and that continuous flow has the potential to produce large-scale amounts of RNA. The first set of experiments in this chapter was done to obtain flux data for the set-up using pure water, water and beads, and the transcription reaction. The results are compared with those found in Chapter II to determine how the transcription reaction affects the membrane and the transmission of RNA. The final set of experiments was designed to continuously produce RNA within the reactor by studying the rate of RNA production, long term RNA production, and the possibility of injecting polymerase throughout the reaction to optimize RNA production. Finally a model of RNA production in the stir-cell has been compared to the experimental data found in these studies with the goal of predicting production characteristics.

5.2 Materials and Methods

5.2.1 Determination of Flux: Pure Water, Beads, and Transcription Reaction

The setup used in these experiments is shown in Figure 5.1. The Amicon Model 3 stir-cell sat atop a magnetic stir plate (Thermolyne Nuova II Stirrer) at a setting of 2, which corresponds to about 125 to 150 revolutions of the stir-bar per minute. The pump was a Cole Parmer MasterFlex Model 7520-35 with an Ismatec Quick Couple Eight-Channel Pump Head. The tubing was Ismatec three-stop, color-coded autoanalysis tubing made of Tygon® with an ID of 0.25 mm. The pump was set at a speed of 0.85, which corresponds to about 1.0 mL/hr but this value can vary by as much as +/- 0.2 mL/hr. The pump created a pressure difference across the membrane and causes the permeate to flow through the tubing. The permeate was then collected on a Mettler AE50 Analytical Balance with the Option O12 Data Interface which sent data to the computer. These data were collected and analyzed. Three sets of experiments were conducted using this set-up. The first was designed to

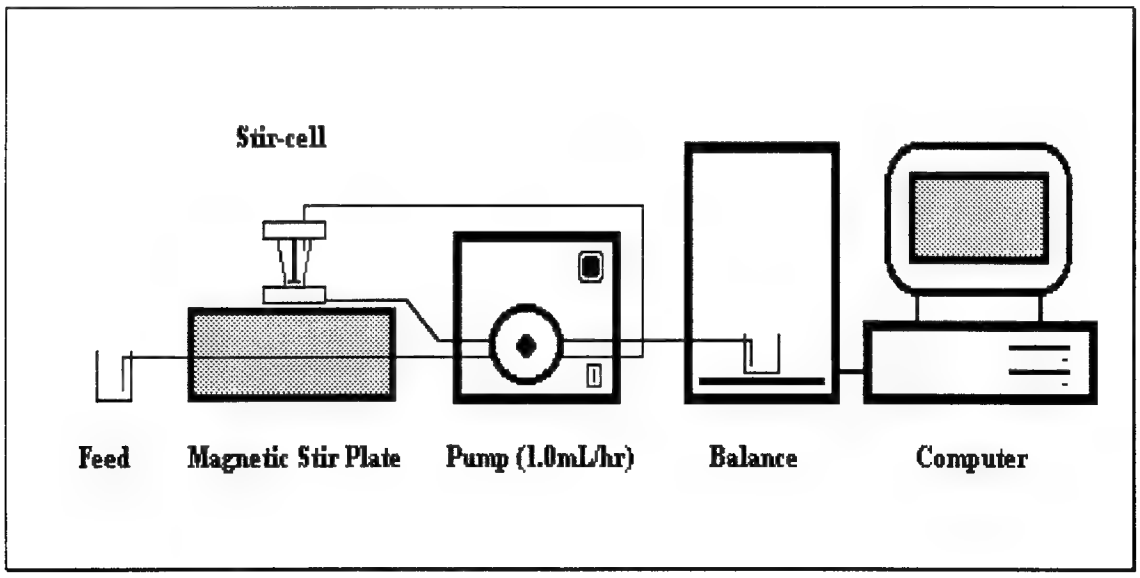


Figure 5.1: Continuous RNA production set-up.

measure the pure water flux to determine a base line for flux. The Amicon YM100 membrane was first washed in d_AH_2O. This was done for one hour, changing the water every twenty minutes. The membrane was then inserted in the stir-cell and 1.0 mL of water was placed in the reactor. The pump was started, the balance was tarred, and every 300 seconds, the computer received a timepoint from the data interface device. A feed line containing fresh d_AH_2O was connected to the pump and delivered fresh water at a rate of 1.0 mL/hr.

The second experiment was a reaction with the agarose beads containing DNA templates and water to determine if the beads become attached to the membrane. This is to follow up on the results obtained from Chapter II. The same setup was used as described above except 200 μL of a 50% strepavidin coated agarose beads solution with biotinylated DNA template attached was added to the stir-cell. For the first 8000 seconds, pure water data were taken. The beads were then added to the stir-cell. Data points were then collected for 7000 seconds. The stir-bar was then stopped and the collection of data continued. The reason for stopping the stir-bar was to assure sedimentation of the beads and to determine the effects of this on flux. The final set of experiments was done by placing a transcription reaction into the stir cell and taking mass measurements over a 400 minutes time interval. The transcription reaction contained the materials described in section 4.2.1.

5.2.2 Rate of Continuous Production of RNA

A rate experiment was accomplished using the continuous flow set-up. A YM100 membrane was first washed for one hour and then placed in the stir-cell. An

addition of 3.0 mL of d_AH_2O was added to the reactor and the membrane was continuously washed with a flow rate of 2 mL/hr. This step was done to equilibrate the membrane and to wash the membranes pores of any remaining preservatives. These preservatives are designed to allow the membrane to be stored and transported. A transcription reaction mixture was set up as described in section 4.2.1 and added to the reactor. In addition, a feed vessel which contained 2.4 mL of NTPs, buffer, and d_AH_2O was set up. The concentrations of NTPs and buffer were consistent with the starting concentration in the reactor. Limiting NTP concentrations were used to get a better signal, with the knowledge that using an optimized concentration will lead to less aborts and more specific product (Chapter IV). Labeled UTP was then seeded in the reaction mixture as well as in the feed vessel. The concentration of labeled UTP was again constant throughout the reaction. A zero timepoint was taken from the retentate side when the labeled UTP was added, then polymerase was added to begin the transcription reaction. At 5, 15, 30, 45, 60, 75, 90, 105, 120, and 135 minutes, 20 μL samples were removed from the retentate. The permeate was collected in a vial and, after each retentate sample was taken, 20 μL from the permeate vial was taken. The permeate vial accumulated the permeate samples and from this collection vial the 20 μL permeate sample was removed at the same interval as the when the retentate samples taken. These samples were added to 15 μL of 75% formamide and loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager.

5.2.3 Continuous Production of RNA

A continuous transcription reaction was set up in the stir-cell. The procedure was similar to that described in section 5.2.2 except a 4.8 mL feed vessel containing NTPs, buffer, and d_AH_2O was used for the longer transcription reaction. Timepoints were taken at every 30 minutes up to 300 minutes by removing 20 μL of retentate using a pipette. The permeate samples were collected in a vial. After each 30 minute time interval the collection vial was changed with a empty vial. From the permeate vials, 20 μL was removed at the end of each 30 minute timepoint. These samples were added to 15 μL of 75% formamide and loaded onto 20% (19:1 crosslinking) denaturing (8M urea) polyacrylamide gels for electrophoresis (600 Volts, 15 milliamps, for 3 hours). The gels were dried and then exposed on the phosphor plate. The plate was developed on the PhosphorImager.

5.2.4 Pulse Injection of Polymerase

A continuous transcription reaction was set up in the stir-cell. The procedure was similar to that described in section 5.2.3. The continuous production of RNA is designed to retain the polymerase so that it is recycled. The previous experiments had only added polymerase at the beginning of each experiment. From the data collected in Chapters III and IV, the polymerase appeared to be the cause of the plateau seen in the production of RNA. These experiments were designed to include a 20 μL pulse injection of polymerase (0.23 mg/mL concentration) at the 120 and 240 minute timepoints to determine the effects of RNA production. Twenty microliter samples

were removed at every 30 minutes for 300 minutes. The retentate samples were removed with a pipette and the permeate samples were withdrawn from the collection vials. These collection vials were filled with permeate flow until the end of a thirty minute time interval, then replaced with a new vial. The permeate sample therefore contained the concentration of RNA from each thirty minute time interval.

5.3 Results and Discussion

5.3.1 Flux Determination

The results from the flux experiments showed that the setup being used was unable to quantitatively show fouling of the membrane. Since the setup uses a pump to draw fluid across the membrane, flux will remain constant. The pump was set at a constant speed, and any fouling of the membrane would cause an increase in the pressure drop to compensate for the membrane fouling. Only after considerable fouling occurred would the pressure drop became so large that the pump could not deliver constant flow. A more useful measurement would be to chart pressure drop versus time, but because the tubing was so small it was impossible to put a pressure gauge at the permeate side of the membrane.

The pure water flux can be seen in Figure 5.2. From the data, the flux appears constant, which is expected from the setup used for these experiments. The initial flux increase is probably due to the leftover preservative located on the membrane during storage.

Figure 5.3 shows the results of putting beads into the reactor. This test was done to confirm the results found in Chapter II. The addition of beads to the reactor should show a decrease in flux. Unfortunately, the setup used will only show flux decline when considerable fouling occurs. The results in Figure 5.3 show that no significant change in flux occurred when the beads were added. The slight increase, after beads were added might represent an increase in pressure drop caused by the pump because of fouling, but no conclusion can be drawn from this procedure. When the stir-bar was stopped, flux did start to decline after about 5000 seconds.

Figure 5.4 reveals the effects of flux when a transcription reaction is performed in the bioreactor. In this case fouling occurs, because at the later timepoints in the transcription reaction experiments the flux begins to slowly decline. This means that although the pump is trying to compensate for the fouling by increasing the pressure

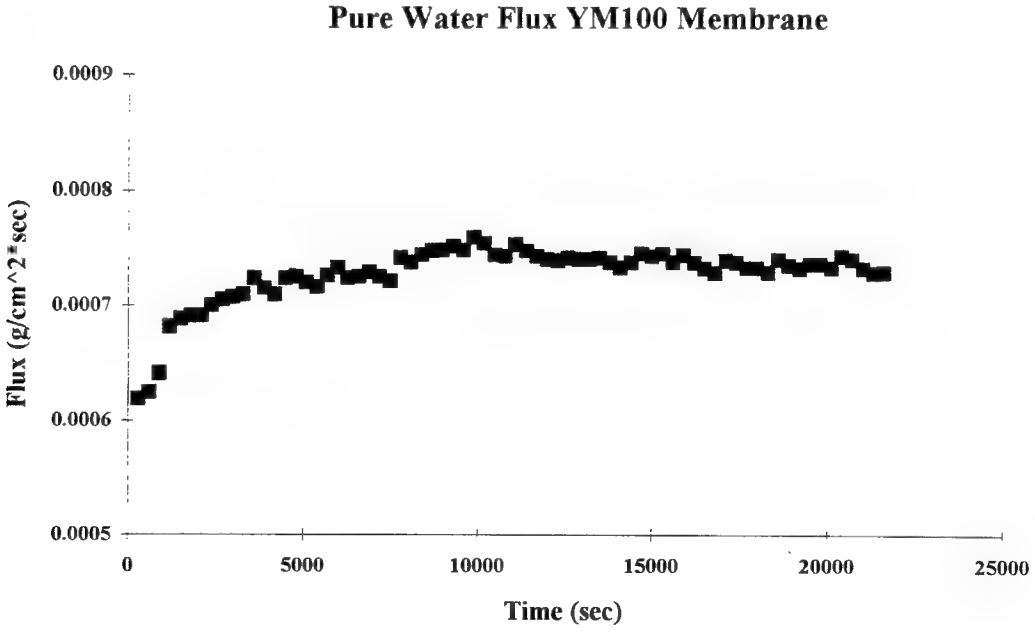


Figure 5.2: Pure water flux revealing a constant flux which occurs because of the pump configuration.

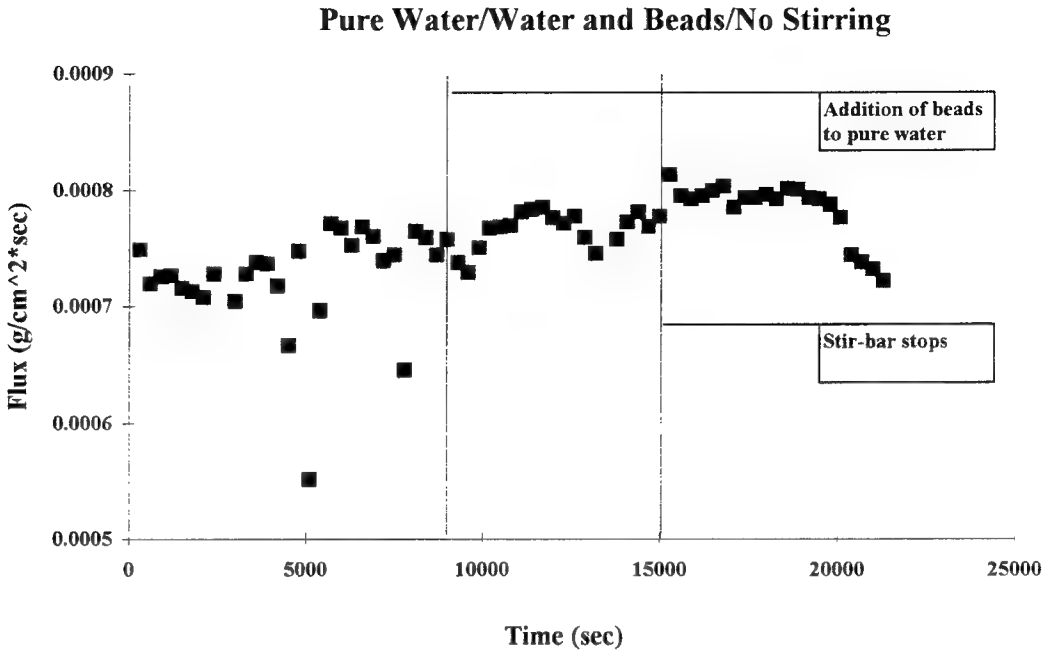


Figure 5.3: Pure water flux compared to the addition of beads and then finally with the stir-bar stationary.

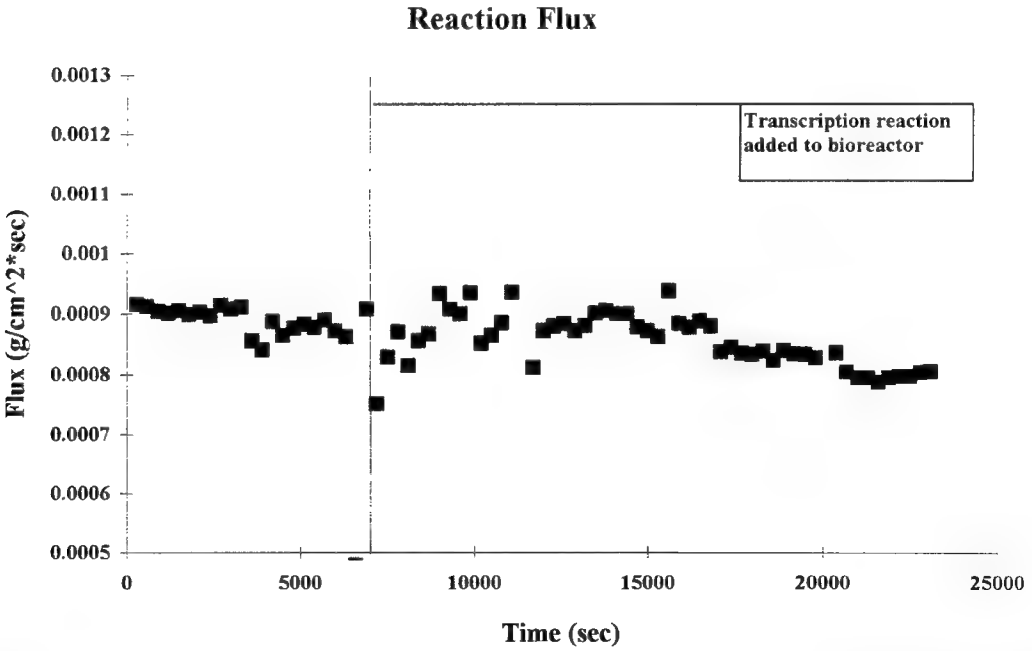


Figure 5.4: The addition of the transcription reaction on the flux of material. The slight decline is caused by the fouling of the membrane and the pump unable to increase the pressure drop to keep flux constant.

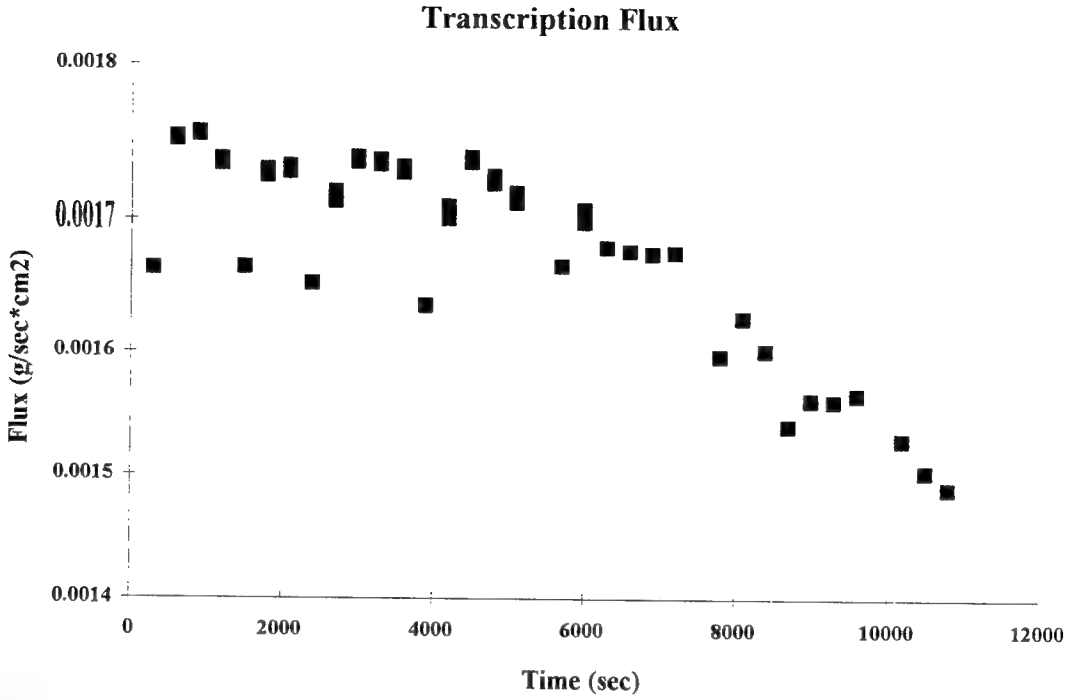


Figure 5.5: The decline in flux is seen when a transcription reaction is performed in the reactor.

drop, it can not do so completely. Once this occurs the flux begins to decline which indicates that fouling is occurring in the membrane. An additional transcription experiment can be seen in Figure 5.5. In these reactions, the pump speed is different.

5.3.2 Model of Continuous Production of RNA

A model has been developed to predict the concentration of RNA produced in the continuous bioreactor. A mass balance is done on the bioreactor with no RNA being introduced to the reactor and assuming a rate of production per unit volume, (r) along with a constant RNA transmission coefficient (T) for the membrane. The resulting equation describes the production of RNA in the bioreactor:

Rate In - Rate Out + Rate Formed = Rate Accumulated

$$Q * 0 - Q * C_{out} + r = V \partial C / \partial t$$

The assumptions are that $r =$ constant (zero-order kinetics, for reactants in excess), and $C_{out} = T * C$. The variables represent flow rate, Q (mL/min), volume, V (mL), time, t (min), concentration in permeate, C_{out} (μM), and concentration in the reactor, C (μM). The rate of production (r) is nanomoles/mL*minute. Eliminating the terms and simplifying, the governing differential equation is as follows:

$$\frac{\partial C}{\partial t} = r - \frac{QTC}{V}$$

Using the initial condition of C = 0 at t = 0 and letting τ = V/(QT), then the resulting solution found using an integration factor yields:

$$C = \tau r \left(1 - e^{-t/\tau}\right)$$

Figure 5.6 is one solution from the model equation with τ = 88.2 min and r = 0.0129

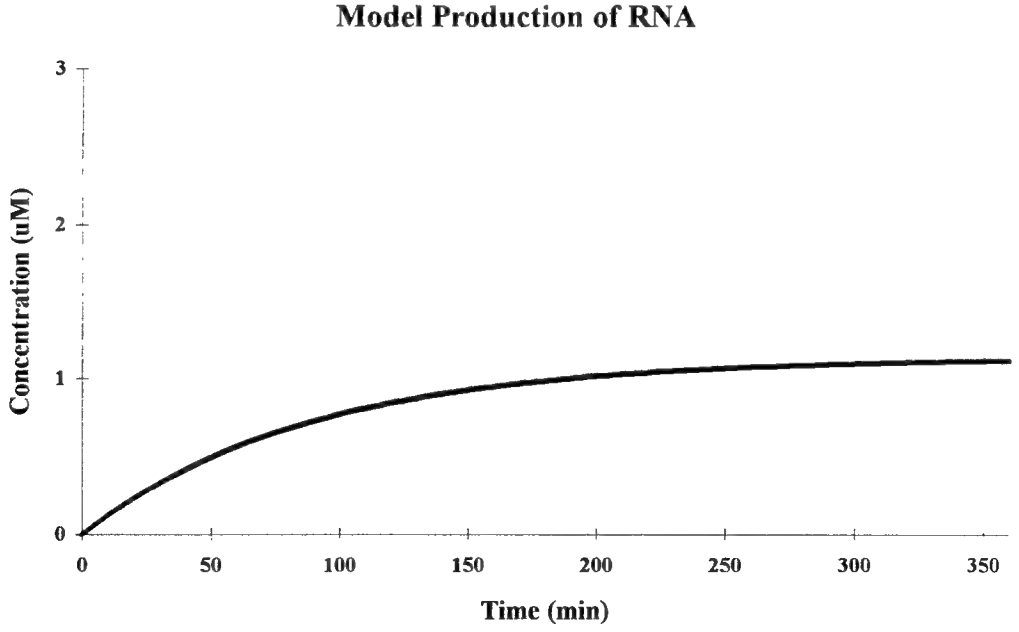


Figure 5.6: Concentration profile in the reactor from the model. The parameters are τ = 88.2 min and r = 0.0129 nanomole/mL*min.

nanomole/mL*min. For short times, the concentration in the reactor builds up due to production of RNA. As the product is transmitted through the membrane, its concentration rise slows down. As $t \rightarrow \infty$, then the concentration reaches a steady state value. In Figure 5.6, this steady state value is 1.14 μM.

5.3.3 Rate of Continuous Production of RNA

The rate of RNA production in the stir-cell bioreactor using a continuous flow reactor is seen in Figure 5.7. The amount of RNA produced is lower initially because of the dead space the permeate flow must travel once it exits the reactor and flows into the collection vial. The rate of RNA production is similar to the results seen in the semi-continuous batch results in Chapter IV (Figure 4.2). The rate of continuous production is 0.0129 versus 0.0115 nanomoles/mL*min in the batch reactions. The

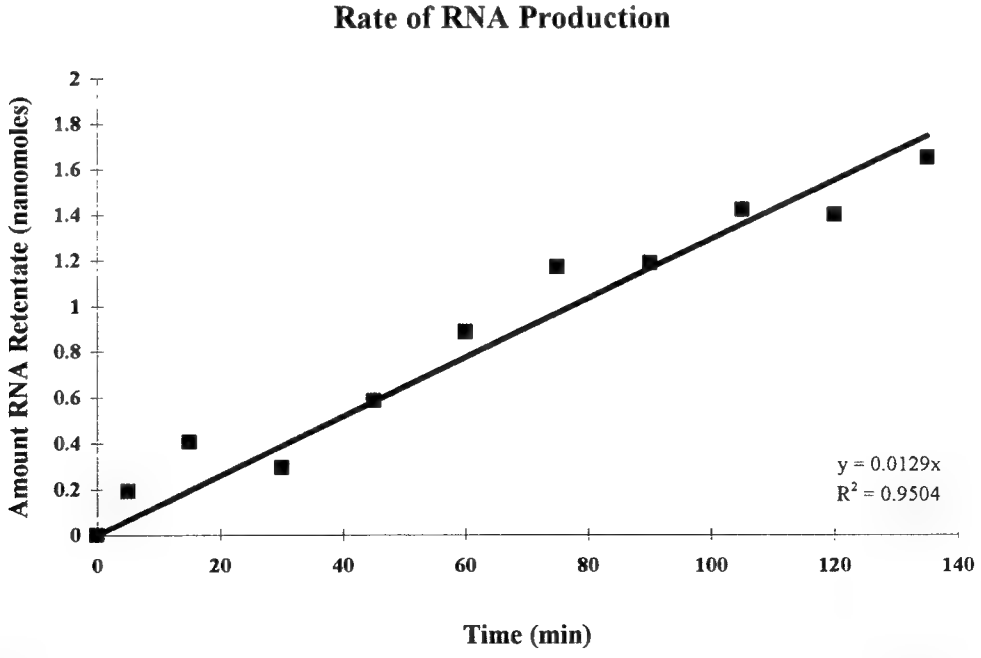


Figure 5.7: Rate of RNA production in the stir-cell bioreactor. The rate of 12-mer RNA production is .0129 nanomoles/mL*minute found by linear regression of the data points.

semi-continuous stir-cell reactions have a rate of 0.0024 nanomoles/mL*min which is about five times slower.

5.3.4 Continuous Production of RNA

Long term production was also studied to determine the feasibility of potential scale-up using a continuous-flow stir-cell bioreactor. Figure 5.8 is a gel, which shows retentate and permeate patterns for a 300 minute production run. Longer runs were not considered due to the large amounts of NTPs and buffer solutions needed for the feed streams. The gel shows that after the first hour the specific RNA (12-mer) production reaches a constant. Additionally, an increase in labeled UTP can be seen. This build up is caused by the polymerase no longer transcribing at the same rate, and thus a build up of NTPs occurs. This build up of NTPs could also be caused by the fouling of the membrane; however, the transmission is practically 90-100% in the below reaction. The membrane also shows considerable radioactive counts which is either labeled UTP or RNA.

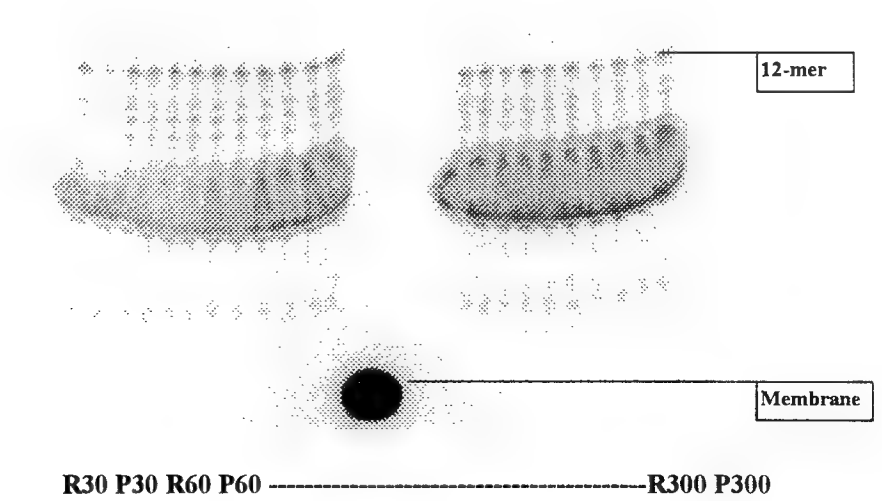


Figure 5.8: Electrophoretic gel of the 300 minute production run in the stir-cell bioreactor. The numbers refer to minutes with R being the retentate at that time and P being the permeate samples.

Figure 5.9 represents, in graphical form, the results of continuous RNA production in the stir-cell bioreactor along with the model which was fitted to the results. The data indicate that production of specific RNA (12-mer) once again reaches maximum in about 90 minutes then begins to decrease. This decrease is due to the reaction rate falling off with time or perhaps the deactivation of polymerase with time. Fouling is occurring, but the retentate and permeate values are very close, indicating that very little RNA is being retained by the membrane. The early permeate timepoints may differ from the model because of the dead time of the permeate flow (0.85 mL/hr) through the tubing (29.92 mm^3) to reach the collection vial. This dead time is equivalent to about 2.0-2.5 minutes, which is relatively small. Figure 5.10

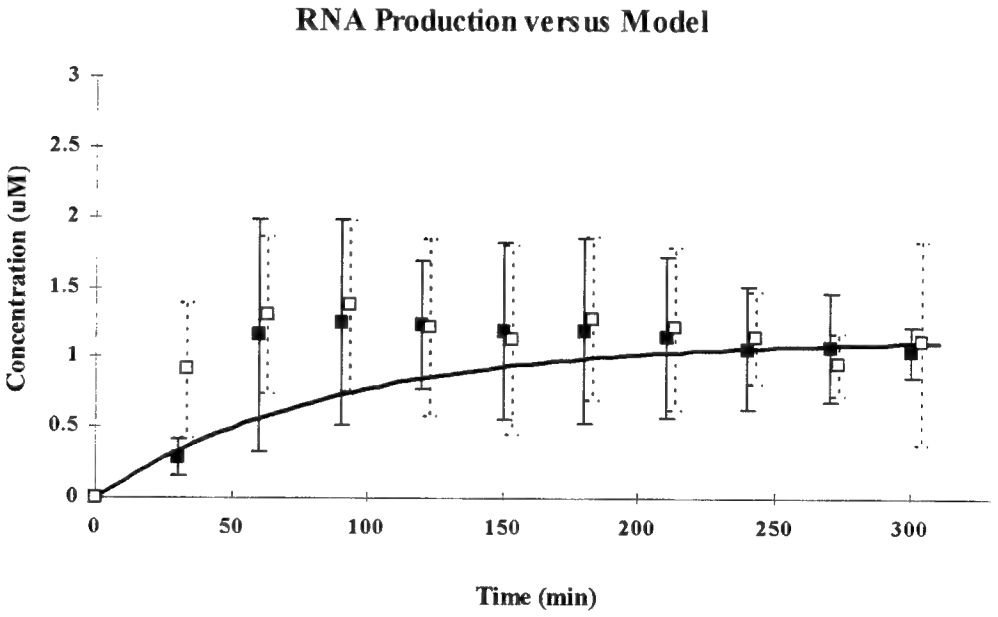


Figure 5.9: Concentration profile of RNA concentration during production in the stir-cell bioreactor. The experimental data are fitted with the model and is represented by the thick line. The permeate samples are the dark squares with the retentate samples represented by the light squares. Error bars represent plus or minus one standard deviation of three runs.

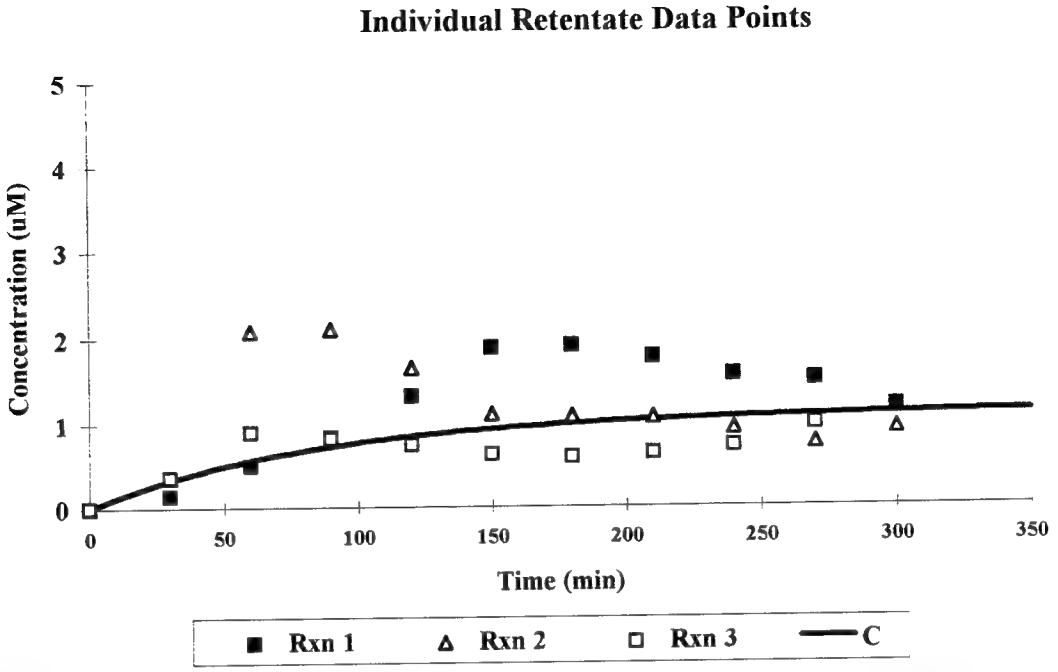


Figure 5.10: Individual retentate data points from the results in Figure 5.9. The model prediction for the retentate concentration from the results in Figure 5.9 is also shown.

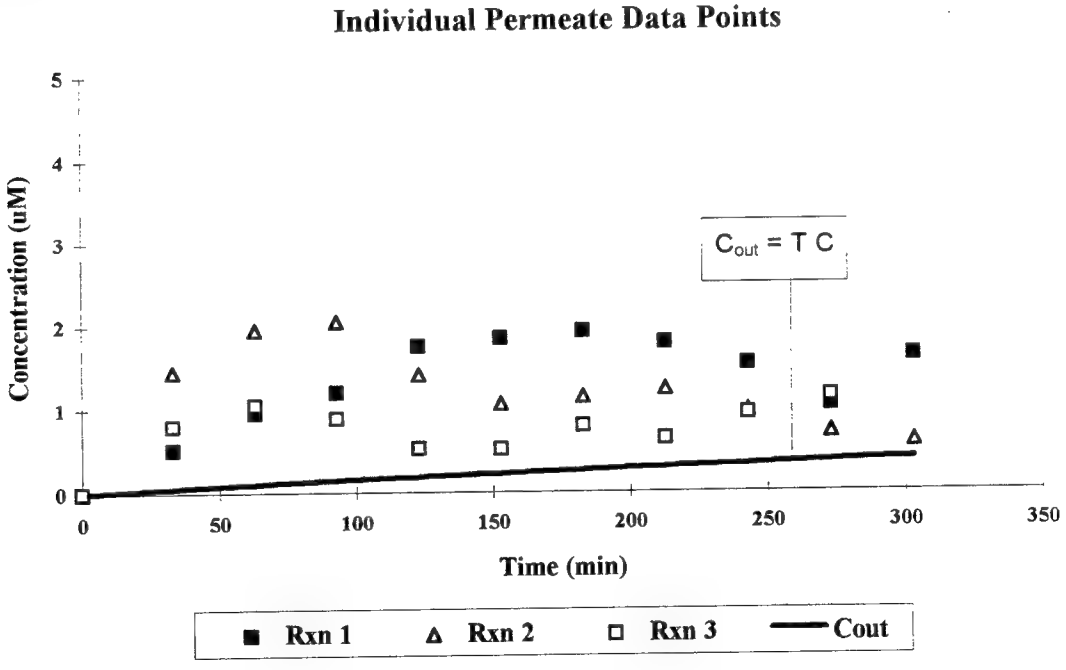


Figure 5.11: Individual permeate data points from the results in Figure 5.9. The model prediction for the permeate concentrations from the results in Figure 5.9 is also shown.

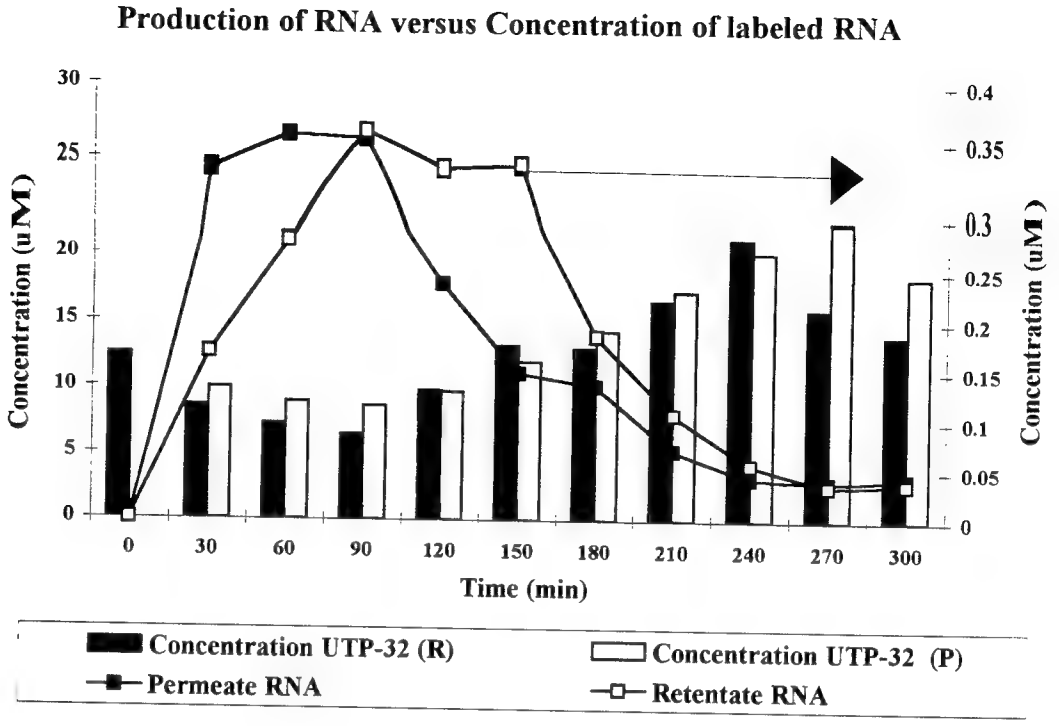


Figure 5.12: Graph representing the production of RNA with the amount of unused labeled UTP in the reactor.

charts the production of RNA in comparison to the concentration of unused labeled UTP. What can be seen is that with RNA production the UTP is used but at later timepoints the rate of RNA production decreases which corresponds to an increase in UTP.

The experimental data were used to determine the validity of the model. From the experimental data, a value for transmission coefficient (T) was chosen to be 0.80, indicating that 80 percent of the RNA is transmitted through the membrane. This amount was comparable to the values found in Chapter II; however, the values in Chapter II were not constant with time, which the above model assumes. The concentration values from the experiment show that the earlier timepoints are significantly higher than what the model predicts. The permeate and retentate values

are about equal with the model in the later timepoints when the reaction reaches a steady state. The reason that the earlier experimental data are much higher in the first 200 minutes is probably due to the fact that the rate is not constant, but rather starts at a higher value then used and then declines. In addition it has been shown in Chapter II that transmission varies with time. The values r and τ were determined by experiment. In Figure 5.9, the transmission coefficient was set at 0.80, which yields $\tau = V/(QT) =$ 88.2 min. From section 5.3.3, the reaction rate was determined to be r = 0.0129 nanomoles/mL*min in a 1.0 mL reaction volume. This reaction rate is compared to the results in Table 4.1 with semi-continuous data which show that the continuous rate of production is equal to the batch rate of production (0.0115 nanomoles/mL*min). The value of C = 1.14 μM is the predicted steady-state value of the product concentration in the reactor. The observed product concentration agrees well with this value.

Finally, for comparison, a reaction was done using the optimized NTP concentrations as described in Chapter IV. The gel can be seen in Figure 5.13 with Figure 5.14 showing the graph of RNA production comparing optimal NTP

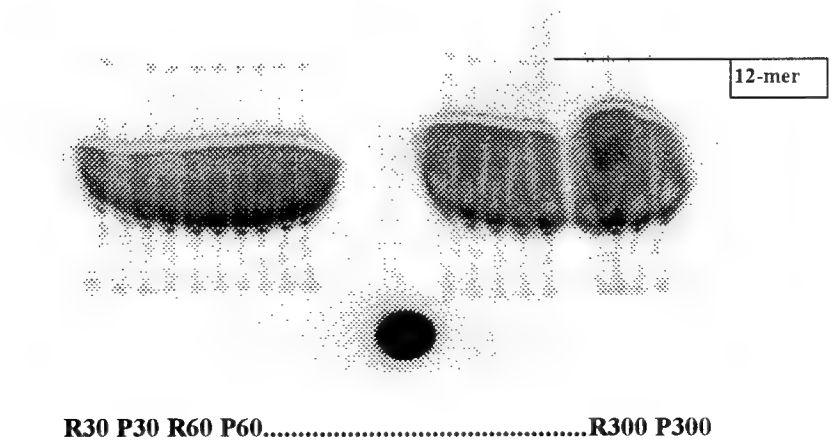


Figure 5.13: Electrophoretic gel of continuous RNA production using optimized NTP concentrations which significantly decreases the amount of aborts while increasing the yield of RNA produced.

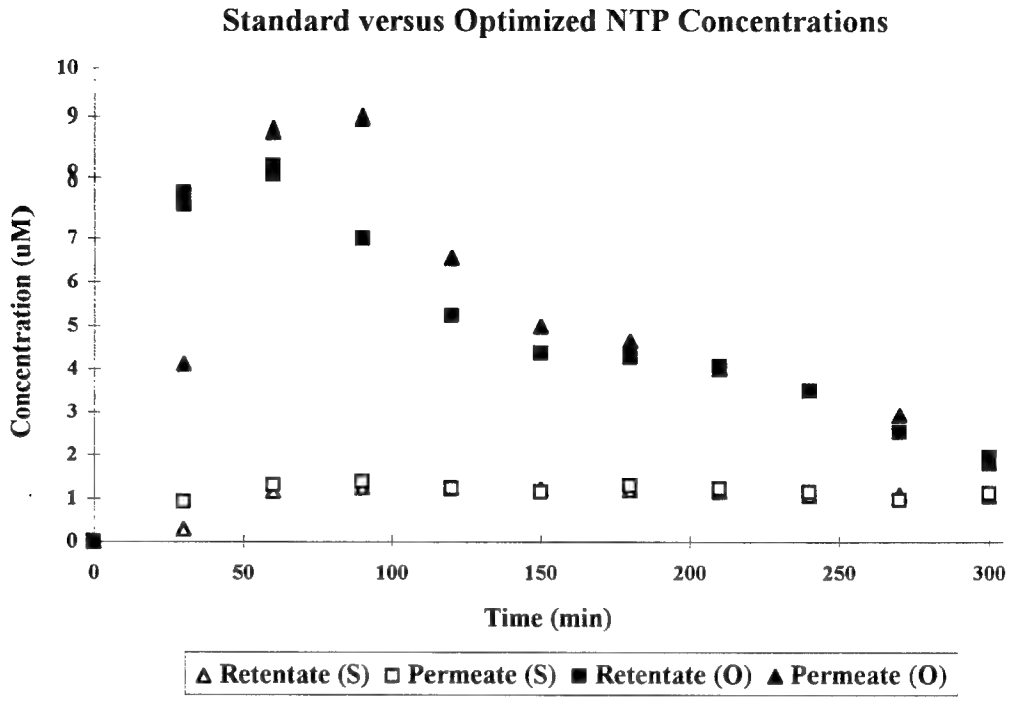


Figure 5.14: Optimized (O) versus standard (S) NTP concentrations. The concentration as well as the amount of RNA produced is greater for optimized NTP concentrations, but the productivity decreases with time for this case.

concentrations with standard NTP concentrations. Using optimized NTP concentrations decreases the amount of aborts and increases the yield. Unfortunately, the productivity falls off with time.

The experimental data were again used to determine the validity of the model developed for the continuous production of RNA. Figure 5.15 shows the optimized production of RNA and the model comparison. The transmission for this experiment was again set at 0.80, and the volume of 1.0 mL, and flow rate of 0.85 mL/hr results in a value of 88.2 min. No independent rate data were obtained using the optimized conditions, but from the experimental data, a value of $C = \tau r = 8.0\ \mu M$ was set as the steady state concentration value. Using this value, a rate of $r = 0.0906$ nanomoles/mL*min is attained. This value is about seven times faster than the rate

obtained using standard NTP concentrations. Semi-continuous studies indicated a value of about ten times faster. The model does not fit the optimized data as well as the standard NTP reactions. For a better fit for both models, the rate of production is probably not a

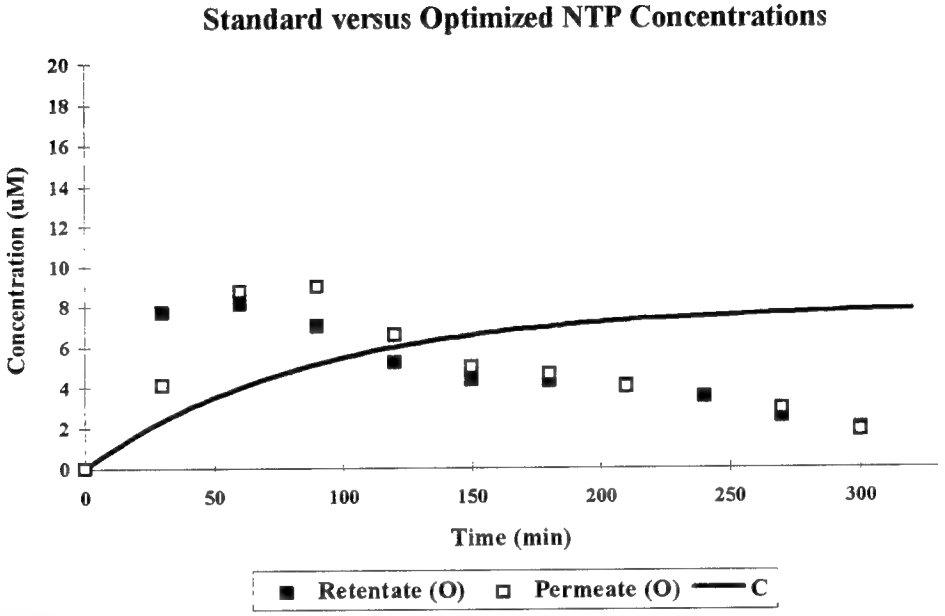


Figure 5.15: Continuous production of RNA using optimized NTP concentrations, model and experiment.

constant, (perhaps due to polymerase deactivation) and is dependent on the concentration of NTPs.

5.3.5 Pulse Injection of Polymerase

The pulse injection experiment did not produce similar results as seen in section 4.3.7, specifically Figure 4.15. The semi-continuous reactions showed that by adding polymerase every 90 minutes an increase in amount of RNA is seen. However in the continuous experiment with standard NTP concentrations, the addition of

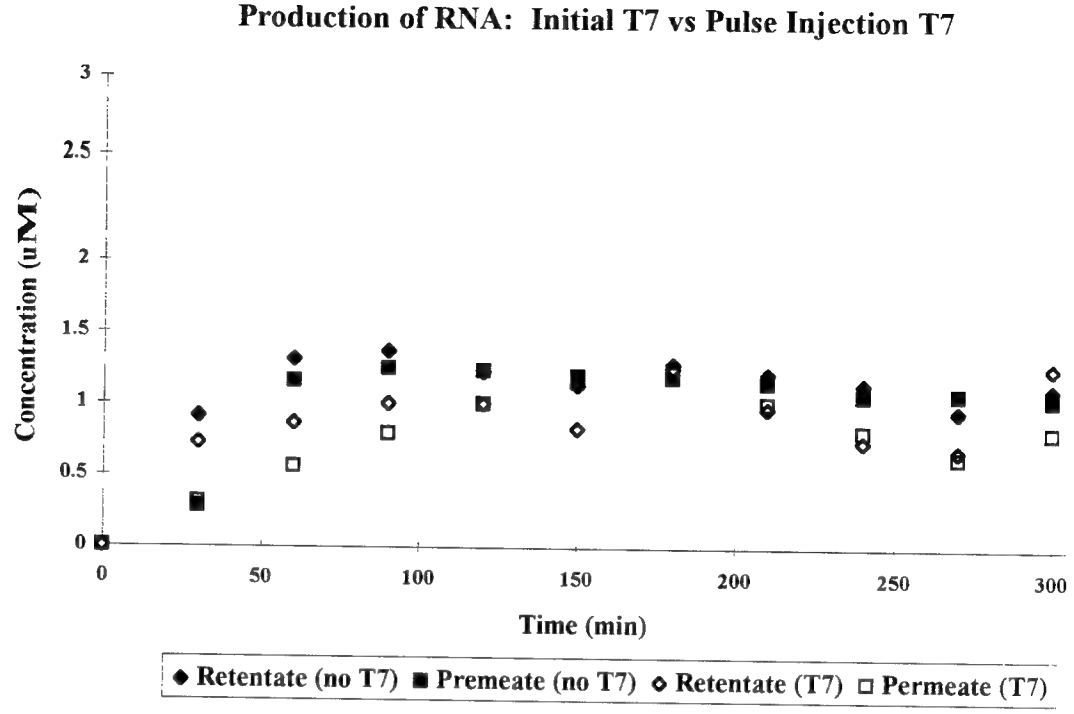


Figure 5.16: Results from the pulse injection experiment which reveals that the addition of polymerase with time for the continuous flow reactor does not increase RNA production as seen in the semi-continuous experiments.

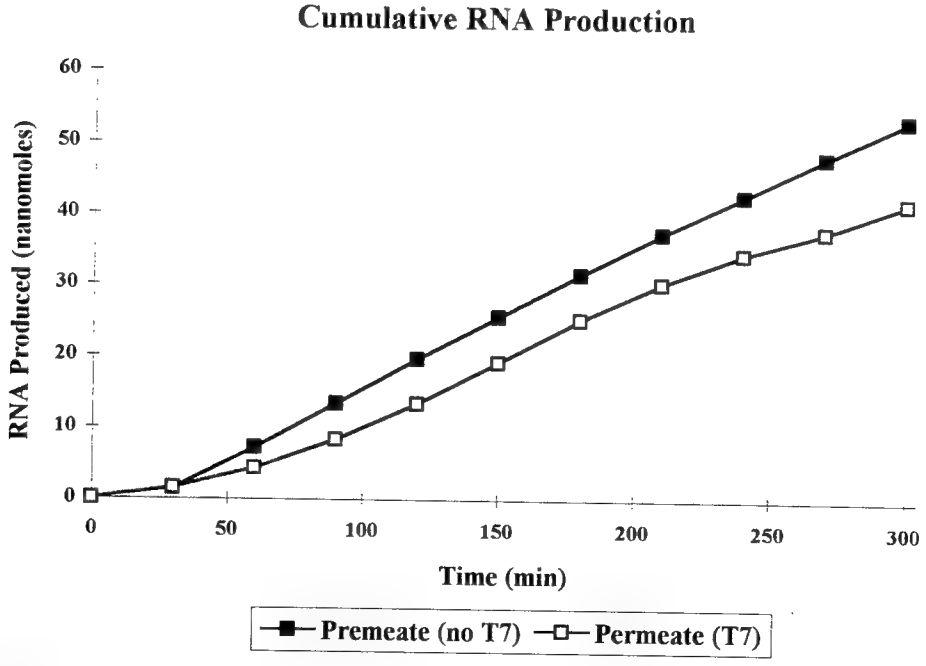


Figure 5.17: Cumulative production of RNA with no additional polymerase added after the first amount (no T7) and with polymerase added after the 120 and 240 minute mark (T7).

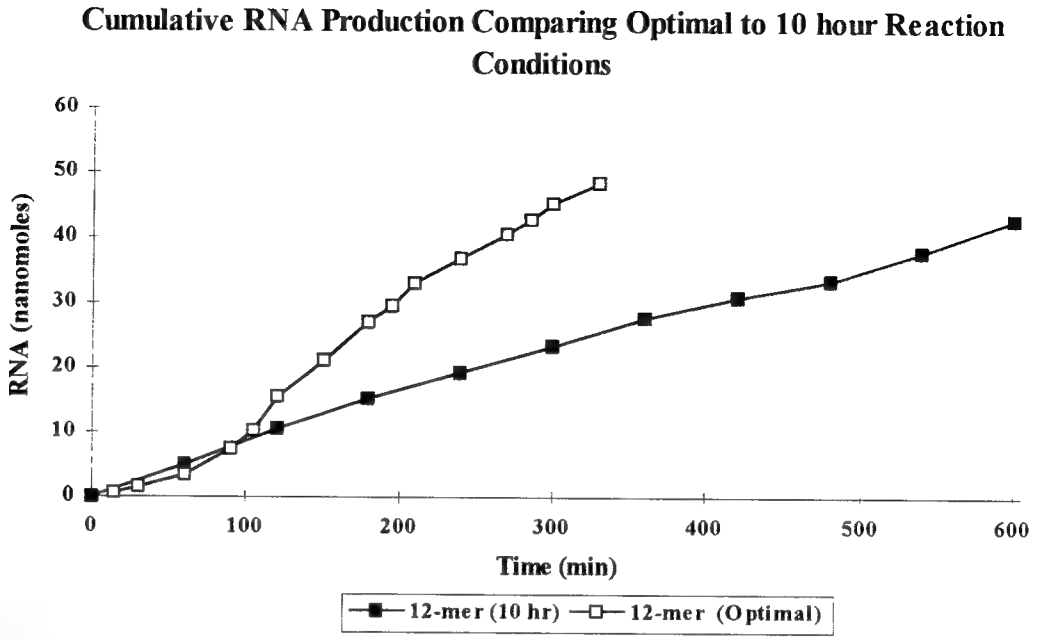


Figure 5.18: Results from Chapter IV which compares the semi-continuous reactions over a 10 hour period and using an optimal injection of polymerase to optimize RNA production.

polymerase at the 120 and 240 minute mark does not yield any more RNA than what is seen if no polymerase is added at these later timepoints. Figure 5.16 compares the results of the pulse injection experiment with the results in the continuous experiment (Figure 5.9). The cumulative amounts of RNA produced can be seen in Figure 5.17. For comparison the results from Chapter IV are found in Figure 5.18. What the data indicate is that the semi-continuous method with polymerase additions every 90 minutes produces about the same amount as the continuous flow reactions in which polymerase is only added in the initial start-up. This indicates that the continuous flow reactor is effective in producing the same amount of RNA as the semi-continuous system but with one fourth the amount of polymerase. Table 5.1 shows the amount of specific (12-mer) RNA made in each system studied and the time required to produce these amounts.

Table 5.1
Continuous RNA Production in the Stir-cell Bioreactor versus Batch Reactions and Semi-continuous Stir-cell Reactions using Standard NTP Concentrations

Reaction		Amount 12-mer Produced (nanomoles)
Stir-cell	Continuous	54 (300 min)
	Semi-continuous	43 (600 min)
	Optimized Semi-continuous	50 (300 min)
Batch		38 (600 min)

5.4 Conclusions

The continuous flow stir-cell bioreactor is an effective alternative for producing RNA. The flux data indicate that in the later timepoints fouling does occur since the flux begins to decline. Unfortunately, with the system design it is impossible to further define this fouling. A model has been developed which closely predicts the actual production of RNA in the stir-cell. This production of RNA has been shown for up to 300 minutes, and shows that peak production occurs in the first 90 to 120 minutes with a slow decrease in production in the later timepoints. Transmission of the RNA is 80-100%, indicating that the membrane is effective in separating the RNA transcripts from the transcription reaction. Finally the pulse injection of polymerase throughout the reaction does not appear to increase the production of RNA. This production of RNA in the continuous mode is equal to the semi-continuous reaction in which polymerase was seeded throughout the reaction.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Overall, a continuous stir-cell membrane bioreactor using immobilized DNA templates for the large-scale, low-cost production of RNA appears to be feasible and deserves further attention. Several important conclusions can be made from the results of this study. First of all, the transcription reaction mixture affects the transmission of RNA through the membrane. This was seen in Chapter II although transmission was not affected as severely in the continuous experiments in Chapter V. Over time this retention of RNA in the bioreactor increases. This retention is due to fouling of the membrane by the reaction materials or the agarose beads sedimenting to the membrane's surface and blocking the pores. Flux data indicates that beads cannot be the definite cause of this fouling. The beads do have the ability to adsorb NTPs and RNA. Data indicate that with time more NTPs are adsorbed to the beads. This adsorption could be a plus for the continuous flow system in that the beads need to be retained somewhat in the reactor so that a reaction can occur. The polymerase denatures over time in the bioreactor, indicating that this could be one reason for the plateau in RNA transcription. In semi-continuous reactions, it was shown that the

addition of polymerase causes more RNA to be produced. It was also shown that in a 10 hour semi-continuous reaction even with additions of NTPs and buffer the reaction still plateaus and levels off. Only when polymerase is added does the production of RNA begin to increase again. Furthermore, the use of an optimized NTP concentration can increase the amount of specific RNA produced as compared to standard or limiting NTP concentrations. Finally a model has been developed which predicts the production of RNA in a continuous fed stir-cell bioreactor. The production of RNA in a continuous flow bioreactor has shown that it produces RNA up to 300 minutes and that the amount produced is equal to the amount produced in the semi-continuous system when polymerase was added throughout the reaction.

There are several areas for improvements in this study. The studies rely on the incorporation of radioactively labeled UTP into the specific product (12-mer) to determine concentrations and amount of RNA made. Since this method was used throughout the studies the results are consistent but the actual amount of RNA made during the reactions could be more or less. There are also inherent difficulties in using labeled UTP because of the need to use limiting NTPs to get a decent signal to be able to quantify the data. Data in this study have shown that more aborts are prevalent when the limiting NTPs are used. Data also support the idea that the freshness of the materials used in the transcription reaction play a direct role in the amount of specific RNA made. Because of this, the measurements in this study are probably an underestimate of the true amount of specific RNA that could be made in the reactor.

Further studies need to be done concentrating on the deactivation of polymerase to determine whether or not the enzyme is the culprit for the plateau seen

in RNA transcription. The reactions done in this study have shown that even with continued addition of NTPs and buffer the reaction continues to decline with time. To produce RNA effectively and cheaply, the polymerase needs to be recycled and remain active in the bioreactor for long periods of time.

Finally the continuous set-up could be changed to produce more RNA. In these experiments the Amicon Model 3 stir-cell is used, but it is not designed to be a bioreactor. The stir-cell is designed for separating and concentrating proteins, not for producing RNA. If the polymerase is deactivated by the stirring action then an alternative to a stir-bar is needed. In addition if the beads are sedimenting to the surface of the membrane then possibly the membrane could be placed vertically in a bioreactor rather than horizontally. The immobilization could also be changed to reduce the adsorption of reaction materials and possible fouling, by having the template immobilized to the surface of the bioreactor rather than in free solution. Furthermore, the design of the continuous system must take into the account the volumes of permeate that are collected. In these studies after a 300 minute reaction close to 6 mL of permeate was collected. This permeate contains unincorporated NTPs, buffer, abortive transcripts, pyrophosphates, and the desired RNA product. One suggestion is to place another stir-cell in sequence with the bioreactor. The permeate from the production bioreactor is fed into the second stir-cell in which a smaller MWCO ultrafiltration membrane is inserted. This membrane would then separate the unincorporated NTPs and buffer and recycle them back into the production bioreactor. The aborts and desired RNA molecules would then be concentrated. Once concentrated this RNA solution could be loaded onto a

chromatography column, and the desired RNA could be separated and purified from the aborts and other impurities. This continuous process would incorporate several steps but should produce and purify large amounts of RNA. The immobilization scheme is designed to produce only one type of RNA molecule. Fortunately, the DNA template could be heated which would cleave the bottom coding strand. Once this strand is removed, the addition of a new strand for a different RNA molecule can be annealed to the immobilized top strand. This system allows for the production of any type of RNA molecule desired and is not limited to one RNA species.

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